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14. ABSTRACT: Type 1 diabetes is considered an autoimmune disease characterized by the presence of inflammatory cells in the islets of Langerhans. These cells are T lymphocytes, considered responsible for the destruction of the insulin producing beta-cells present in the islets. When the majority of the beta cells are dead, the disease presents, frequently with an abrupt and clinically serious onset. The aim of this program is to determine whom among the Army personnel is at high risk to develop the disease in order to prevent the unexpected onset of the disease that may be associated with tragic consequences, and to initiate an educational program aimed at reducing practical and psychological hurdles. Furthermore, different individuals develop disease complications (i.e., retinopathy, nephropathy, neuropathy) at different timepoints after the onset. The susceptibility to complications could also be genetic. The human genome will be scanned systematically to characterize these susceptibility genes. Proteomic analysis will be performed in tandem to confirm the genetic associations.

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INTRODUCTION

Since there are individuals with diabetes that after 10 years from clinical onset still retain healthy kidney, neurological, and vision function while others are already experiencing diabetic complications damaging to these physiologic systems, our working hypothesis has been that there is a genetic contribution associated with increased risk for developing complications of diabetes. Identifying SNP markers linked to these phenotypes will improve understanding of the molecular mechanisms underlying these diseases and enable the development of assays for improved prediction of individuals at highest risk of developing complications. The project will ultimately lead to advances in the early treatment and prevention of life threatening disease. **The overall goal of the research project is to provide a means to genetically test diabetic patients for their inherited risk for developing the 3 principal complications of diabetes, i.e., nephropathy, neuropathy, and retinopathy.** The research aims are to use index cases, recruited from Children's Hospital of Pittsburgh, the University of Pittsburgh Transplantation Institute, and the U.S. Military Healthcare System to ascertain, recruit, and prepare DNA from appropriate members of families in which diabetic complications have occurred. The project goals are to determine whether complications are linked to any of the proposed genomic candidate loci and to compare the genotyping results obtained from the Pittsburgh Cohort Genetic Study of Diabetic Complications with genotyping results from the diabetic complication family trios collected within the U.S. Military, thus confirming the association between gene and phenotype in independently collected cohorts.

The research hypothesis on which our project is based is that there is a genetic contribution linking susceptible individuals to increased risk for developing diabetic complications. That identifying SNP markers linked to these phenotypes will enable improved prediction ultimately leading to advances in the prevention of life threatening disease. The final goal of the entire project will be to provide a means to genetically test military personnel and families for their risk for developing diabetes and its complications.

BODY: GENETIC ANALYSIS OF DIABETIC COMPLICATIONS

Assembled cohorts for genetic research. Single nucleotide polymorphisms (SNPs) are being analyzed for association to the diabetic-nephropathy phenotype. Patterns of allelic inheritance from parents to affected offspring have been evaluated by transmission/disequilibrium testing (TDT) of family trios composed of DNA sample obtained from father, mother, and affected offspring (Ewens and Spielman, 1995). Multiple sources of diabetic-nephropathy families have been collected (Figure 1). These include cases obtained from the Children's Hospital of Pittsburgh collection of extended families, family trios recruited from the University of Pittsburgh School of Medicine Transplantation Institute, and the Juvenile Diabetes Research Foundation collection of diabetic family trios (Morel et al., 1988; Mueller et al., 2006). At present there are 325 T1DN family trios available for this study, including 159 T1DN trios with end-stage renal disease.

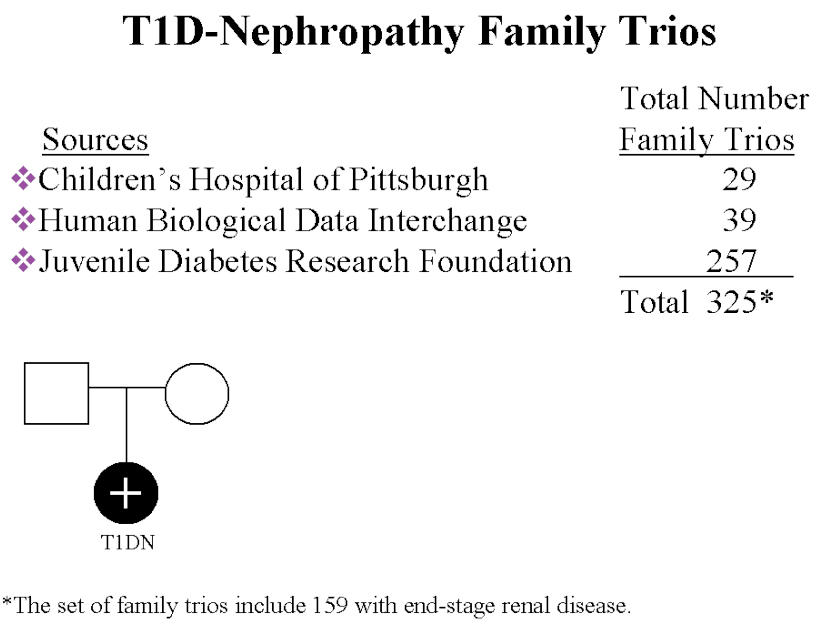

Figure 1. Sources of type 1 diabetic nephropathy (T1DN) family trios collected. Families have been recruited from multiple sources to provide a cohort of 325 families consisting of both parents and offspring with T1DN. Of the 257 families provided through our collaboration with the Juvenile Diabetes Research Foundation the proband in 139 families had end-stage renal disease. Of the families recruited from the Children's Hospital of Pittsburgh 20 out of 29 had end-stage renal disease.

Cohorts comprised of singleton cases and controls have also been recruited (Tables 1 and 2). The cohorts of singletons include approximately 1,200 participants roughly evenly divided between T1DN cases and T1D controls. The set of cases include subgroups with persistent proteinuria and ESRD consisting of 170 and 408 participants, respectively. Control subjects, whether part of the singleton or family trio cohorts, exhibit normoalbuminuria despite duration of T1D exceeding 15 years. The overall genotyping strategy for this phase of the project is to complete the analysis of the candidate loci using the diabetic families that have been collected. The cohorts available for the study are linked with clinical documentation of diabetes, diabetic complications, and related health information. These sources consisting of family DNAs as well as patient health history (e.g., type of diabetes, age of diabetic onset, duration of diabetes prior to onset of nephropathy, as well as history of cigarette smoking) will be used to stratify the cohort in order to better correlate the significance of linkage of a particular locus with the severity of the complication.

Table 1. Case T1D-Nephropathy Singletons and Family Trios Available from the Junvenile Diabetes Research Foundation

	Type 1 Diabetic Nephropathy			
	Singletons	**Family Trios**		
		Proband	Father	Mother
General Information:				
Number of Singletons or Family Trios	578	257		
Sex of Proband (Male)	53%	43%		
Sex of Proband (Female)	47%	57%		
Mean Age at Enrollment (yrs)	43	40	68	66
Ethnicity Caucasian	100%	100%	100%	100%
History of Diabetes:				
Type 1 Diabetes	100%	100%	1%	0.9%
Type 2 Diabetes	0%	0%	12%	12%
Mean Duration of Diabetes at enrollment (yrs)	31	29		
Mean Hemoglobin A1C	7.6%	7.9%	5.9%	5.7%
Taking Insulin	80%	89%	7%	6%
Mean Insulin Dosage/kg Body Weight	0.51	0.57	0.62	0.58
Insulin Regimen <2 Shots	7%	6%	2%	0.9%
Insulin Regimen MDI	53%	59%	5%	5%
Insulin Regimen Pump	17%	21%	0.4%	0.4%
Related Health Staus:				
Mean Body Mass Index	26	26	29	27
Mean Natural Waist Circumference (inches)	35	35	42	37
Mean Systolic Blood Pressure (mm/Hg)	132	130	132	132
Mean Diastolic Blood Pressure (mm/Hg)	74	75	76	75
Mean Total Cholesterol (mg/dL)	188	199	193	213
Mean HDL (mg/dL)	53	56	45	56
Taking Anti-Hypertensive Drugs	61%	53%	48%	40%
Taking Lipid Lowering Medication	47%	41%	32%	25%
History of Smoking	49%	46%	20%	9%
Presence of Other Autoimmune Disease	21%	14%	6%	11%

Pancreas Transplantation	31%	22%		
Evidence of Diabetic Nephropathy:				
Hypertension	71%	74%	52%	52%
Cardiovascular Complication	84%	80%	63%	57%
Mean ACR/AER 1st Urine	1213	1331	122	65
Mean ACR/AER 2nd Urine	1254	1455		
Mean ACR/AER 3rd Urine	1351	1163		
Mean GFR (Cockcroft-Gault) ml/min	61	63	82	75
Mean GFR (MDRD) ml/min/1.73m2	48	48	70	72
Mean Serum Creatinine (mg/dL)	2.2	2.0	1.2	1.0
Mean Serum Cystatin C (mg/dL)	2.2	2.1	1.2	1.2
Presence of Diabetic End Stage Renal Disease:				
End-Stage Renal Disease	66%	52%	0%	0.4%
Mean Time to Event (yrs)	25	25		
Mean End-Stage Renal Disease Duration (yrs)	8	7		
Presence of Diabetic Neuropathy:				
Diabetic Neuropathy	68%	64%	2%	1%
Peripheral Vascular Complication	42%	26%	0.9%	1%
Gangren	8%	6%	8%	6%
Non-Traumatic Amputation	17%	9%		
Presence of Diabetic Retinopathy:				
Pan-Retinal Laser Treatment	81%	78%	3%	2%
Focal Laser Treatment	10%	8%	0%	0%

Table 2. Control T1D Singletons and Family Trios Available from the Junvenile Diabetes Research Foundation

	Type 1 Diabetes without Nephropathy			
	Singletons	**Family Trios**		
		Proband	Father	Mother
General Information:				
Number of Singletons or Family Trios	596	312		
Sex of Proband (Male)	41%	42%		
Sex of Proband (Female)	59%	58%		
Mean Age at Enrollment (yrs)	40	35	64	62
Ethnicity Caucasian	100%	100%	100%	100%
History of Diabetes:				

Type 1 Diabetes	100%	100%	2%	0.4%
Type 2 Diabetes	0%	0%	6%	4%
Mean Duration of Diabetes at enrollment (yrs)	26	24		
Mean Hemoglobin A1C	7.4%	7.5%	5.7%	5.5%
Taking Insulin	100%	100%	7%	6%
Mean Insulin Dosage/kg Body Weight	0.57	0.59	0.50	0.44
Insulin Regimen <2 Shots	5%	4%	1%	0.8%
Insulin Regimen MDI	56%	48%	4%	4%
Insulin Regimen Pump	36%	47%	2%	1%
Related Health Staus:				
Mean Body Mass Index	26	27	30	29
Mean Natural Waist Circumference (inches)	34	33	40	35
Mean Systolic Blood Pressure (mm/Hg)	118	116	130	127
Mean Diastolic Blood Pressure (mm/Hg)	71	71	77	75
Mean Total Cholesterol (mg/dL)	185	186	187	214
Mean HDL (mg/dL)	59	57	45	57
Taking Anti-Hypertensive Drugs	0%	0%	36%	28%
Taking Lipid Lowering Medication	15%	15%	34%	22%
History of Smoking	34%	32%	27%	21%
Presence of Other Autoimmune Disease	21%	22%	13%	17%
Pancreas Transplantation	0%	0%		
Evidence of Diabetic Nephropathy:				
Hypertension	5%	2%	43%	34%
Cardiovascular Complication	7%	7%	51%	40%
Mean ACR/AER 1st Urine	7	7	23	12
Mean ACR/AER 2nd Urine	7	7		
Mean ACR/AER 3rd Urine	9	7		
Mean GFR (Cockcroft-Gault) ml/min	108	114	89	78
Mean GFR (MDRD) ml/min/1.73m2	87	88	74	70
Mean Serum Creatinine (mg/dL)	0.9	0.9	1.1	0.9
Mean Serum Cystatin C (mg/dL)	0.9	0.9	1.1	1.1
Presence of Diabetic End Stage Renal Disease:				
End-Stage Renal Disease	0%	0%	0.8%	0%
Mean Time to Event (yrs)				
Mean End-Stage Renal Disease Duration (yrs)				
Presence of Diabetic Neuropathy:				
Diabetic Neuropathy	14%	8%	2%	0.8%

Peripheral Vascular Complication	3%	2%	1%	1%
Gangren	0.3%	0%	0.3%	0%
Non-Traumatic Amputation	0.5%	0%		
Presence of Diabetic Retinopathy:				
Pan-Retinal Laser Treatment	17%	13%	3%	0.4%
Focal Laser Treatment	2%	1%	0.4%	0%

The cohort of affected child trios was recruited from the patient population enrolled at the University of Pittsburgh Thomas E. Starzl Transplant Institute and Children's Hospital of Pittsburgh. Recruitment of singleton patients and family trios is supervised by Ms. Shelia Fedorek RN Clinical Research Manager at the University of Pittsburgh Medical Center. The proband in each family trios exhibited T1D-ESRD. Recruitment of trios at CHP was governed by the human subjects protocol approved by the University of Pittsburgh Institutional Review Board. Patients were recruited from those who had received transplanted kidney to treat T1D-ESRD. Patients and their family members were asked to consent to providing 10 ml blood obtained by vein puncture as well as a brief medical history relating to onset of T1D, ESRD, and hypertension (Appendix A and B). A second cohort was composed of 139 affected child trios obtained from the GoKinD sample collection (Mueller et al., 2006). The GoKinD cohort was recruited independently of the Pittsburgh cohort by collaborative efforts of the Juvenile Diabetes Research Foundation, National Institutes of Health, and U.S. Center of Disease Control (Mueller et al., 2006). Material from the GoKinD cohort was provided as solutions of DNA, purified from lymphoblastoid cell lines, originating from family trios as well as singletons exhibiting T1D-ESRD.

We are now focusing our collection effort on recruiting an equally large number of family trios from the U.S. Military Healthcare System. We are receiving family trios and singletons recruited at the University of Hawaii Manoa as part of our collaboration with Dr. Richard Arakaki. Patients and their families will also be recruited from Walter Reed Hospital in Washington, DC, pending that institution's IRB approval. Our collaboration at Walter Reed Hospital is with Dr. Robert Vigersky. We anticipate that together these sources will provide an additional 300 family trios of parents and patient for genetic analysis and an equivalent collection of singletons. We intend to recruit participants exhibiting the 3 principal complications of diabetes, i.e., nephropathy, neuropathy, and retinopathy. The prevalence of neuropathy in the U.S. diabetic population is estimated between 60% and 70% and accounts for roughly 80,000 lower limb amputations, greater than half of the total number of amputations performed in the U.S. annually. We anticipate that after 15 years duration of diabetes roughly 22% of diabetic patients experience moderate to severe diabetic retinopathy (Klein et al., 2005). Based on the reported prevalence of diabetic retinopathy and neuropathy it is anticipated that we, and our collaborators, will continue to recruit equally large numbers of family trio cohorts specific for these complications. The recruitment goal is to involve an additional 300 family trios for each diabetic complication into the study. The research plan is to use TDT association analysis to genotype the family trios and by comparing patterns of inheritance between independently collected cohorts confirm association between genetic marker and increased risk of developing disease (Lander and Kruglyak, 1995).

Justification of the proposed sample size for the genetic studies. In order to estimate the strength of the study to detect the proposed genetic effect we have followed the theoretical models proposed by Risch and Merikangas (1996). The TDT and related methods can detect the effect of genes that make a relatively small contribution, provided that susceptibility alleles are in linkage disequilibrium with marker alleles (Table 3). Linkage disequilibrium refers to the genetic situation in which 2 alleles at different loci are transmitted together from parent to offspring more often than would be predicted by random chance. For example, from the results of Quinn et al. (1996) on familial factors that influence the development of type 1 diabetic nephropathy it is possible, in principle, to estimate the magnitude of the effect of a hypothetical single gene. Genetic risk of developing type 1 diabetic nephropathy was observed to increase from 25% to 72%, nearly a 3-fold increase in prevalence, in siblings of persons with type 1 diabetic nephropathy. Genetic modeling of the high rate of incidence observed in families with a history of the disease is consistent with a mode of inheritance that is dominant. Simulations of dominant inheritance models designed to identify allelic polymorphisms linked to the disease marker indicate that between 150 and 300 family trios would need to be genotyped to achieve 80% power when applying the TDT when the marker is present between 10% and 80% in the study population. In contrast, the minimum number of families required to achieve a similar level of statistical confidence using affected siblings would be between 500 and 5,000 families over an identical range of allele frequencies (Table 3). The number of persons that need to be recruited into the study vary with allele frequency, however, the TDT design is beneficial in that fewer numbers are needed to meet the required level of significance (Risch and Merikangas, 1996, 1997; Scott and Rogus, 2000; Mueller et al., 2006). While

we anticipate that many of the SNPs under study will meet these criteria, *a priori* allelic frequency values are not yet available. Despite the possible difficulties in detecting effects by genetic analysis the fact that available sample sizes for the cohorts associated with the collection and those available through our collaboration with the U.S. Military Healthcare System achieve the number appropriate for successful analysis of the genetic risk level, and will continue to grow, the potential benefits for increased knowledge amply justify these studies.

Table 3. Number of Family Trios and Affected Sibling Pairs Needed for Identification of a Disease Gene.

Allele Frequency	Family Trios TDT Analysis	Affected Sibling Pairs ASP Analysis
0.01	1,959	29,807
0.10	251	840
0.20	164	526
0.25	150	508
0.50	150	840
0.75	251	3,243
0.80	307	5,087
0.90	590	20,638
0.99	5,727	2,100,721

Sample size required to achieve 80% statistical power were calculated as described by Risch and Merikangas (1996) using the correction published in Risch and Merikangas (1997). The power calculation assumed a genotypic risk ratio of 3 and a dominant multiplicative mode of inheritance.

The inclusion of case and control singletons along with a cohort of family trios has the advantage that eligible singletons are more easily identified and can be recruited in higher numbers. For diseases such as diabetic nephropathy, in which 38 years of age corresponds to the mean age of ESRD onset, the typical age of the parents is between 60 and 80. Loss of older parents from the study, due to death or declining health that prevents their participation, are principal factors that reduce collection of families and limit use of TDT as an approach to the examination of diseases that strike young populations. Cohorts of singletons provide an important advantage that they can be identified more quickly and studied efficiently in sufficient numbers to provide statistical power to detect genetic association. Genetic analysis of singletons are, however, prone to occurrence of false positive results particularly if population stratification exists between cases and controls. For that reason combined genetic analysis of the case/control singletons and family trio cohorts provide the advantage that outcomes of genetic association can be tested using independent populations, controlling for false positive signals as well as population stratification.

The anticipated outcome of our study has been that statistically significant signals will be observed in case/control singletons and in case family trios while control family trios will be negative indicating a positive association of the candidate gene with the disease. Alternatively, analysis of each of the cohorts will provide results that are not statistically significant providing evidence to rule out the candidate allele as affecting incidence of the disease. Other outcomes are also possible and may indicate possible gene-gene and gene-environment interactions (Scott and Rogus, 2000). In particular, the utility of control trios occurs in situations when the disease is highly prevalent (affecting greater than 30% of the at risk population) and when gene-environment interactions exist (i.e., poor glycemic control or positive history of smoking) there is particular relevance (Scott and Rogus, 2000). Two potential patterns of allele inheritance consistent with environmental influence are: 1) significant association when case/control singletons and control trios are analyzed combined with no association for case trios; and 2) significant association when control trios are analyzed and no association for case/control singletons and case trios (Mueller et al., 2006). Observation of either of these when

combined with analysis of Hb1AC levels, smoking history, or other non-inherited factor will provide evidence for presence of environmental exposure on disease incidence.

Genotyping of T1D-nephropathy candidate genes. Family-based studies have shown that T1D siblings of T1D patients have a 2 to 3 fold increased incidence of nephropathy when the patient exhibits diabetic renal disease. The incidence of diabetic nephropathy in siblings continues to increase when the patient exhibits severe nephropathy, i.e., end-stage renal disease (Seaquist et al., 1989; Quinn et al., 1996; Harjutsalo et al., 2004). Our working hypothesis is that there is a genetic contribution associated with increased risk for developing T1D-nephropathy. Identifying SNP markers linked to the phenotype will enable improved prediction of individuals at highest risk of developing T1DN ultimately leading to advances in the early treatment and prevention of life threatening disease. The overall goal of the research project is to provide a means to genetically test T1D patients for their inherited risk of developing diabetic end-stage renal disease. The experimental approach follows a process of preparing DNA from blood samples of patients recruited by clinicians at the Thomas E. Starzl Transplantation Institute, the University of Hawaii Manoa, and at Walter Reed Hospital. Recruitment of patient families is followed by genotyping SNPs that have been identified previously through our collaboration with Richard Spielman's laboratory at the University of Pennsylvania as well as from other research groups publishing their findings in the scientific literature (Ewens et al., 2005; Rich, 2006).

TDT linkage analysis has been performed using allelic transmission data obtained by genotyping 121 candidate genes in 92 family trios examined so far. A summary of the genotyping data obtained from 5 of the 92 family trios are listed in Figure 2.

Genotyping Data for T1D-ESRD Family Trios

		Affected Child Trios T1D-ESRD (N=17)														
Locus Symbol	dbSNP ID	7001-01	7001-02	7001-03	7003-01	7003-02	7003-03	7004-01	7004-02	7004-03	7009-01	7009-02	7009-03	7016-01	7016-02	7016-03
BCL2	rs1481031	T/T	C/T	T/T	C/C	C/C	C/C	C/T	T/T	T/T	C/C	C/C	C/C	C/T	T/T	T/T
BCL2	rs2062011	A/T	A/T	A/A	T/T	T/T	T/T	A/T	A/T	A/A	T/T	T/T	T/T	A/T	A/T	A/T
CAT	rs1049982	G/G	G/A	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/A	G/G	G/A	G/G	G/G	G/G
CAT	rs560807	T/T	T/A	T/A	A/A	A/A	A/A	T/T	T/A	T/T	A/A	A/A	A/A	T/A	A/A	T/A
CD2AP	rs923146	A/G	A/G	G/G		G/G						G/G	A/G	A/G	A/A	A/A
CDH3	rs1111721	G/A	G/G	G/G	A/A	G/A	G/A	A/A	G/A	G/A	G/A	G/A	G/A	G/G	G/A	G/G
COL4A1	rs679062	G/G	A/G	G/G	A/G	A/A	A/G	A/G	A/A	A/G	A/A	A/A	A/A	A/G	A/G	G/G
COL4A1	rs614282	A/A	G/A	A/A	G/A	G/G	G/A	G/A	G/G	G/A	G/G	G/G	G/G	G/A	G/A	A/A
FAT1	rs462422	C/G	G/G	C/G	C/G	C/C	C/G	C/G	G/G	G/G	C/G	C/G	C/C	C/G	C/G	C/C
FAT1	rs997696	G/G	G/G	G/G	C/G	G/G	C/G	G/G	C/G	G/G	G/G	G/G	G/G	C/G	C/G	G/G
GPX1	rs1800668	G/G	G/G	G/G	G/G	G/A	G/G	G/A	G/A	G/G	G/A	G/A	A/A	A/A	G/A	A/A
HNF1B1/TCF2	rs2688	A/C	A/C	C/C	C/C	A/C	A/C	A/C	A/C	C/C	A/C	A/C	A/C	C/C	C/C	C/C
KIRREL	rs912572	A/A	A/A	A/A	C/A	C/A	C/A	C/C	A/A	C/A	C/A	A/A	A/A	A/A	A/A	A/A
KIRREL	rs927662	C/T	C/T	C/C	T/T	C/T	C/T	C/C	C/T	C/T		C/T	C/T	C/T	C/T	C/T
LAMA4	rs3734287	T/T	T/T	T/T	T/C	T/C	T/T	T/C	C/C	T/C	T/T	C/C	T/C	T/T	C/C	T/C
LAMC1	rs10797819	A/A	G/G	A/G	G/G	A/A	A/G	A/G	A/G	G/G	G/G	A/G	A/G	A/G	G/G	A/G
LAMC1	rs2296288	G/A	G/G	G/G	G/G	A/A	G/A	G/A	G/A	G/G	G/G	G/A	G/A	G/A	G/G	G/A
LAMC1	rs4652775	T/T	A/A	A/T	A/A	T/T	T/A	A/T	A/T	A/A	A/A	A/T	A/T	A/T	A/A	A/T
LAMC1	rs7410919	A/A	G/G	A/G	G/G	A/A	A/G	A/G	A/G	G/G	G/G	A/G	A/G	A/G	G/G	A/G
LAMC1	rs7556132	A/A	G/G	A/G	G/G	A/A	A/G	A/G		G/G	G/G	A/G	A/G	A/G	G/G	A/G
LPL	rs13702	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/C	T/T	T/T	T/C	T/C	T/C
LPL	rs320	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/G	T/T	T/T	T/G	T/G	T/G
LPL	rs326	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/C	T/T	T/T	T/C	T/C	T/C
MMP9	rs11697325	G/G	G/G	G/G	G/A	G/A	G/A	G/A	A/A	G/A	G/A	G/G	G/G	G/A	G/A	A/A
NPHS1	rs437168	A/G	G/G	G/G	G/G	G/G	G/G	A/G	G/G	A/G	G/G	G/G	G/G	G/G	G/G	G/G
NPHS1	rs466452	A/G	G/G	G/G	G/G	A/G	A/G	A/A	A/G		G/G	A/G	A/G	G/G	G/G	G/G
NPHS2	rs1079291				G/G	G/T	G/T	G/G	T/T	G/T	G/T	G/T	T/T	G/G	G/G	G/G
NPHS2	rs12406197				C/C	C/T	C/C	C/T	C/C	C/T	C/T	C/C	C/C	C/C	C/T	C/C
p22phox/CYBA	rs4673	T/C	T/C	C/C	C/C	C/C	C/C	C/C	T/C	C/C	C/C	C/C	C/C	C/C	T/C	C/C
PRKCB1	rs1015408	A/T	A/T	A/A	T/T	T/T	T/T	T/T	T/T	T/T	A/T	A/A	A/T	T/T	A/T	T/T
SLC2A4	rs5435	A/A	G/A	G/A	G/G	G/A	G/A	G/A	G/A	G/A	G/G	G/A	G/G	G/A	G/A	G/A
SMAD3	rs12594610	T/C	T/C	T/C	T/T	T/C	T/C	T/T	T/T	T/T	T/C	T/C	T/C	T/C	T/T	T/C
SMAD3	rs4776890	T/T	G/T	T/T	G/G	G/T	G/T	G/T	G/T	G/G	G/T	T/T	T/T	G/T	G/T	T/T
TGFBR2	rs6792117	T/C	C/C	C/C	C/C	C/C	C/C	C/C	T/T	T/C	T/T	T/C	T/T	T/C	T/C	T/C
USF1	rs2516839	C/T	C/T	C/T	T/T	T/T	T/T	T/T	C/C	C/T	T/T	C/T	T/T	C/T	T/T	T/T

Heterozygous parents are indicated in yellow

Figure 2. Summary of the genotyping data obtained from 5 of the 92 family trios. Each family trio contained an index case with T1D and end-stage renal disease. The identifying number of the family and participant as well as the locus symbol and SNP number are indicated. Yellow shaded genotypes indicate that a parent was heterozygous for the genetic variant.

At present there are 9 genetic loci that when assayed by TDT linkage analysis exhibit values exceeding the minimum p-value of 0.05 and 4 genetic loci exceeding a p-value of 0.005 (Figures 3 and 4). Among these loci, the most significant linkages observed, p-values less than 0.003, encode the proteins B-cell Lymphoma Protein 2, Collagen Type IV Alpha 1 Chain, Lipoprotein Lipase, and SMA-MAD related protein 3 indicated by the locus symbols BCL2, COL4A1, LPL and SMAD3, respectively. The genomic regions identified in our study have also been linked to a variety of complications-related ailments. For example, the BCL2 protein may affect kidney morphogenesis by negatively influencing inductive interactions between epithelium and mesenchyme, important processes during kidney organogenesis (Nakayama et al., 1994). Type IV collagen has been implicated in T1DN due to its altered expression in the glomerular basement membrane of the kidney during fetal development and organogenesis (Van Agtmael et al., 2005; Fumo et al., 1994). In patients with DN triglyceride levels are increased possibly as a result of decreased LPL activity. LPL hydrolyzes triglycerides and is a rate limiting step in postprandial utilization of fatty acids. Genetic variants of Lipoprotein Lipase have been implicated in presence and severity of microalbuminuria in diabetic patients (Mattu et al., 2002). SMAD3 plays a central role as a mediator of TGF-beta signaling and subsequent production of fibrotic matrix, including increased synthesis of type IV collagen and glomerular basement membrane thickening correlated with proteinuria (Isono et al., 2002; Fujimoto et al., 2003; Guh et al., 2003; Flanders, 2004).

Candidate Loci for Diabetic Nephropathy

p-value < 0.005

B cell CCL-lymphoma 2 (Chr 18q21.3)

rs1245770	Intron
rs2062011	Intron
rs1481031	Intron

Collagen4A1 (Chr 13q34)

rs614282	Intron
rs679062	Intron

Lipoprotein lipase (Chr 8p22)

rs320	Intron
rs326	Intron
rs13702	3'UTR

SMAD3 (Chr 15q21-q22)

rs4776890	Intron
rs12594610	Intron

p-value <0.05

Catalase (Chr 11p13)

rs1049982	5'UTR
rs560807	Intron

Cytochrome b-254 (Chr 16q24)

rs4673	His(C)72Tyr(T)

Glutathione peroxidase (Chr 3p21.3)

rs1800668	5'UTR

Laminin, alpha 4 (Chr 6q21)

rs3734287	Intron

Laminin, gamma 1 (Chr 1q31)

rs10797819	Intron
rs4652775	Intron
rs2296288	Cys(C)182Cys(T)
rs7556132	Ile(A)458Val(G)
rs2296292	Ala(C)592Ala(A)
rs20557	Asn(C)837Asn(T)
rs7410919	Leu(T)888Pro(C)

Matrix Metalloproteinase 9 (Chr 20q11.2-q13.1)

rs11697325	Intergenic 8.2kb 5'

Protein kinase C, beta 1 (Chr 16q11.2)

rs1015408	Intron

TGF beta receptor II (Chr 3p22)

rs6792117	Intron

Transcription Factor 2 (Chr 17cen-q21.3)

rs2688	Intron

Figure 3. Preliminary TDT analysis of 121 loci using 92 family trios identified 13 loci with p-values of 0.05 or better. The name of each loci, its chromosomal location, SNP reference sequence number (e.g., rs1245770), and functional effect (e.g., whether it is associated with a coding change or is found within an intron) are indicat

Significant T1D-Nephropathy Markers

Locus	RefSeq	Allele	Tran	Total	p-value
Highly Significant: (p-value < 0.005)					
BCL2	rs2062011	A/T	50	73	0.0016
COL4A1	rs679062	C/T	46	67	0.0023
LPL	rs13702	C/T	46	67	0.0023
LPL	rs320	G/T	48	72	0.0047
LPL	rs326	A/G	52	78	0.0032
SMAD3	rs12594610	A/G	24	74	0.0025

Figure 4. The top 6 genetic markers are linked to 4 loci when data obtained from our cohort of 92 family trios are analyzed. The loci BCL2, COL4A1, LPL, and SMAD3 are linked to markers with p-values exceeding 0.005. The SNP reference sequence number, identity of the alleles observed, and their transmission rates are indicated.

Genetic analysis of glomerular basement membrane proteins for linkage to T1D-nephropathy. Candidate loci used for TDT analysis of T1DN were chosen based on the results of previous studies associating regions of the human genome to the phenotype and have implicated proteins expressed within the kidney. For example, the extracellular matrix protein encoded by the loci COL4A1 (p-value 0.002) comprises a major component of glomerular basement membrane during organogenesis forming the blood-urine barrier in the developing kidney. Transmission of alleles linked to the COL4A1 locus has been studied for 92 family trios (Figure 5). TDT statistical analysis supports the hypothesis for highly significant genetic linkage of COL4A1 to the nephropathy phenotype.

Candidate Loci within the Glomerular Basement Membrane

Gene Symbol	Description	dbSNP ID	Alleles		Trans	Non-Trans	Total	% Trans	Chi-Sq	p-value
Glomerular Basement Membrane:										
COL4A1	Collagen Type IV	rs679062	C/T	T	46	21	67	69	9.3	0.0023
COL4A4	Collagen Type IV	rs614282	C/T	C	24	44	68	35	5.9	0.0153
LAMA4	Laminin Alpha-4	rs3734287	C/T	C	26	45	71	37	5.1	0.0241
LAMC1	Laminin Gamma-1	rs10797819	G/A	A	53	34	87	61	4.1	0.0416
LAMC1	Laminin Gamma-1	rs2296288	C/T	T	55	34	89	62	5.0	0.0260
LAMC1	Laminin Gamma-1	rs4652775	A/T	A	35	53	88	40	3.7	0.0550
LAMC1	Laminin Gamma-1	rs7410919	T/C	T	55	34	89	62	5.0	0.0260
LAMC1	Laminin Gamma-1	rs7556132	A/G	A	53	32	85	62	5.2	0.0227
MMP9	Matrix Metalloproteinase 9	rs11697325	A/G	A	42	20	62	68	7.8	0.0052

Chi-square and p-values for Slit-Diaphragm loci will be reported once the total number of transmissions exceeds N=50.

Figure 5. Summary of genetic transmission data for loci associated with the glomerular basement membrane. The gene symbol, gene description, and identifying number of each SNP are indicated. The geentic transmission data for each allele includes the number of observed major allele transmissions from heterozygous parent and the total number of transmissions. The percentage of transmissions as well as the chi-square and p-values are indicated.

Other loci whose gene products are associated with function of the glomerular basement membrane have been studied as part of our candidate gene approach and include matrix metalloproteinase-9 (MMP9), alpha integrin (ITGA3), and nephrin (NPHS1) (Figure 6). Analysis of the ITGA3 and NPHS1 loci indicated no linkage to T1DN but alleles of the MMP9 loci have provided evidence for linkage, exhibiting a p-value 0.006. MMP9 activity has been implicated in glomerulosclerosis models of kidney disease and has been in regulation of podocin a principal component of the glomerular slit diaphragm (Gerke et al., 2005).

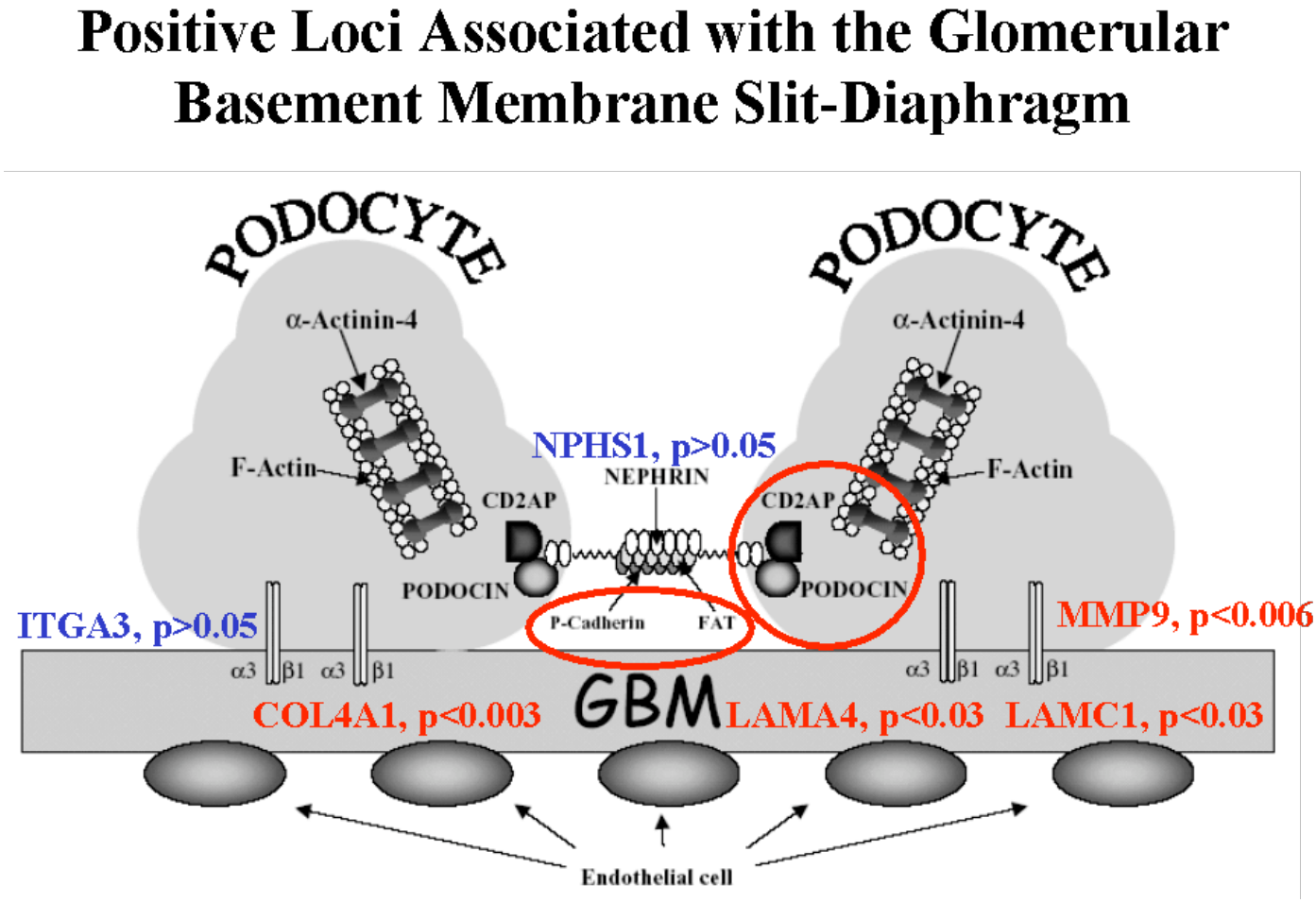

Figure 6. Organization of the blood:urine boundary in the kidney. The glomerular basement membrane (GBM) is shown along with its relation to the endothelial cell layer, podocyte cell, and slit diaphragm. Proteins comprising the GBM are encoded by loci COL4A1, LAMA4, and LAMC1. Loci linked to the T1DN phenotype are indicated in red along with their associated p-values. Loci shown in blue have been tested by TDT analysis but are not linked to the phenotype. The loci circled in red are being examined during the current research period. The figure was modified after the illustration published by Vincenti and Ghiggeri (2005).

New assays are underway in order to evaluate the possibility that other proteins making up the glomerular basement membrane and slit-diaphragm may correlate to disease prevalence (Figure 7). SNP typing assays have been implemented for the podocyte expressed proteins podocin (NPHS2), CD2-associated protein (CD2AP), p-cadherin (CDH3), and homolog of FAT tumor suppressor (FAT) proteins comprising the slit diaphragm. SNP markers are being examined in our collection of families in order to complete the analysis of the set of glomerular basement membrane proteins, and the integrated glomerular slit diaphragm. Our goal is to access the possible role of these loci in genetic risk to T1DN.

Candidate Loci within the Slit Diaphragm Candidate Loci

Gene Symbol	Description	dbSNP ID	Alleles		Trans	Non-Trans	Total	% Trans	Chi-Sq	p-value
Slit-Diaphragm:										
CD2AP	CD2 Associated Protein	rs923146	A/G	A	3	4	7	43	---	---
CDH3	Cadherin 3	rs1111721	G/A	A	5	11	16	31	---	---
FAT1	FAT Tumor Suppressor	rs462422	C/G	C	10	5	15	67	---	---
FAT1	FAT Tumor Suppressor	rs997696	C/G	C	1	5	6	17	---	---
KIRREL	Nephrin-Like 1	rs912572	C/A	A	5	4	9	56	---	---
KIRREL	Nephrin-Like 1	rs927662	C/T	T	7	8	15	47	---	---
NPHS1	Nephrin	rs437168	A/G	A	1	2	3	33	---	---
NPHS1	Nephrin	rs466452	A/G	A	5	7	12	42	---	---
NPHS2	Podocin	rs1079291	C/T	T	4	3	7	57	---	---
NPHS2	Podocin	rs12406197	G/T	T	11	4	15	73	---	---

Chi-square and p-values for Slit-Diaphragm loci will be reported once the total number of transmissions exceeds N=50.

Figure 7. Summary of genetic transmission data for loci associated with the slit-diaphragm. The gene symbol, gene description, and identifying number of each SNP are indicated. The genetic transmission data for each allele includes the number of observed major allele transmissions from heterozygous parent and the total number of transmissions. The percentage of transmissions as well as the chi-square and p-values are indicated for each SNP.

Genetic analysis of NADPH oxidase associated proteins for linkage to T1D-nephropathy. The kidney expressed form of NADPH oxidase is produced primarily in the kidney cortex and podocytes. Increased activity of the enzyme has been correlated with nephropathy in animal models (Li and Shah, 2003). Conditions that down regulate the activity of NADPH oxidase have been shown to contribute positively to improved renal function. Genetic dissection of the human expressed form of NADPH oxidase is being accomplished by TDT analysis of family trios. Analysis of the transmission data obtained from 92 family trios have implicated the gene product of the CYBA locus, encoding the p22phox subunit, as being correlated with T1DN and exhibiting a p-value of 0.017 (Figure 8). Other subunits of NADPH oxidase are also being investigated. These loci include the p47phox (NCF1), p67phox (NCF2), and the GTP-binding protein (RAC1). Genotyping typing assays have been designed for SNPs at each of these loci and are underway. Analysis of the catalytic subunit NOX4 in the same set of family trios has been completed and exhibited no linkage to the phenotype.

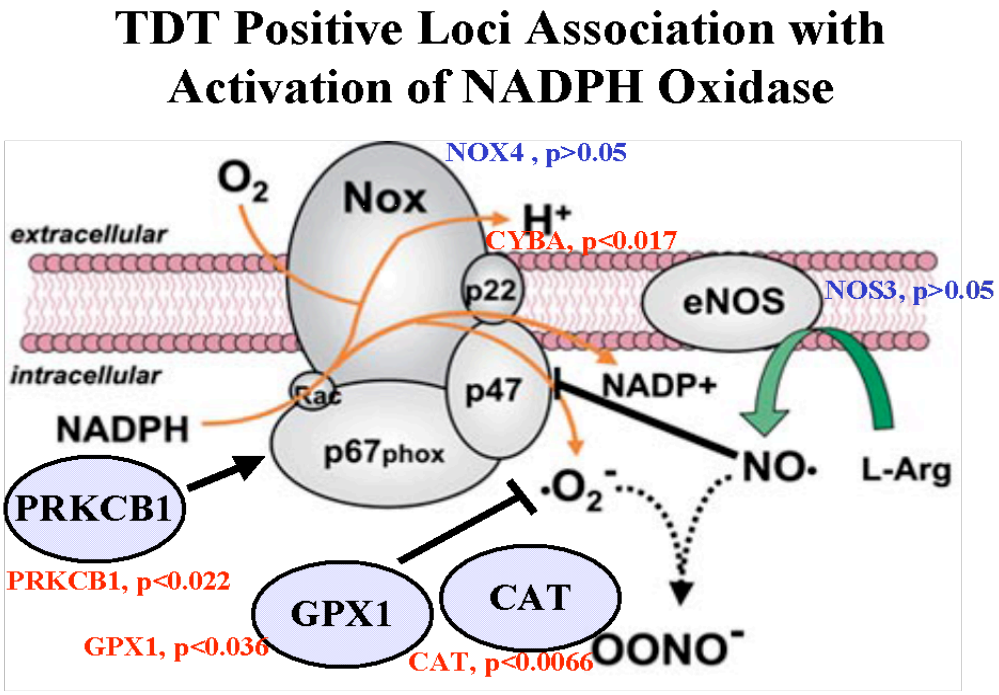

Figure 8. Structure of the NADPH oxidase and organization of the proteins that affect its enzymatic activity. Loci linked to the T1DN phenotype are indicated in red along with their associated p-values. Loci shown in blue have been tested by TDT analysis but are not linked to the phenotype. The figure was modified after the illustration published by Dusting et al. (2005).

Gene products that regulate NADPH oxidase activity are included in our candidate gene approach to understanding the genetic risk associated with T1DN (Figure 9). The catalytic activity of NADPH oxidase utilizes reducing equivalents from NADPH to convert oxygen molecule into super oxide radical. A protein that increases the activity of NADPH oxidase is protein kinase C beta 1 subunit (PRKCB1) while the product of nitric oxide synthase 3 (NOS3) inhibits enzyme activity. The subunit beta-1 of protein kinase C (PRKCB1) has been examined among the cohort of 92 family trios and exhibited a statistically significant linkage to the phenotype, presenting a p-value of 0.022. The enzymatic action of protein kinase C has been associated with increased activity in patients exhibiting nephropathy (Araki et al., 2003). In contrast, the NOS3 locus showed no linkage.

Candidated Loci within the NADPH Oxidase Complex

Gene Symbol	Description	dbSNP ID	Alleles		Trans	Non-Trans	Total	% Trans	Chi-Sq	p-value
CAT	Catalase	rs1049982	C/T	C	27	51	78	35	7.4	0.0066
CAT	Catalase	rs560807	A/T	A	33	52	85	39	4.2	0.0393
GPX1	Glutathione Peroxidase	rs1800668	A/G	G	21	37	58	36	4.4	0.0356
p22phox/CYBA	Cytochrome b(-245)	rs4673	C/T	C	22	41	63	35	5.7	0.0167
PRKCB1	Protein Kinase C, Beta-1	rs1015408	A/T	T	19	36	55	35	5.3	0.0219
NOS3	Nitric Oxide Synthase 3	rs1549758	A/G	A	23	20	43	53	0.2	0.6473
NOS3	Nitric Oxide Synthase 3	rs1007311	T/C	T	28	26	54	52	0.1	0.7855
NOS3	Nitric Oxide Synthase 3	rs1799983	A/C	A	23	21	44	52	0.1	0.7630
NOS3	Nitric Oxide Synthase 3	rs891512	A/G	A	24	21	45	53	0.2	0.6547
NOX4	NADPH Oxidase 4	rs317147	C/T	C	35	33	68	51	0.1	0.8084
NOX4	NADPH Oxidase 4	rs12364595	C/T	C	36	33	69	52	0.1	0.7180
NOX4	NADPH Oxidase 4	rs7925520	C/G	G	34	26	60	57	1.1	0.3017

Figure 9. Summary of genetic transmission data for loci associated with the NADPH oxidase. The gene symbol, gene description, and identifying number of each SNP are indicated. The genetic transmission data for each allele includes the number of observed major allele transmissions from heterozygous parent and the total number of transmissions. The percentage of transmissions as well as the chi-square and p-values are indicated for each genetic variant.

The gene products glutathione peroxidase (GPX1) and catalase (CAT) are components of the super oxide radical scavenging system present in the kidney cortex. Analysis by the transmission disequilibrium test of loci GPX1 and CAT provided evidence for linkage to T1DN with p-values of 0.036 and 0.0066, respectively (Figure 9). Reduced expression of Catalase has been linked to kidney disease in rat models renal insufficiency and fibrosis (Kobayashi et al., 2005; Sindhu et al., 2005).

Web tools for assay design, data management, and sharing of results. In order to enable the genotyping process, pyrosequence-based typing assays have been designed for SNPs associated with each loci of interest. Pyrosequence assays for multiple alleles of each of the loci have been validated for use with the patient samples collected for this project. In order to extend the successful design of assays for the complete set of candidate loci, computer software has been written to provide primers for pyrosequence-based typing assays that are optimized for examining the genetics of diabetic-nephropathy (Figure 10). The software is a web-based program capable of designing assays for genotyping large numbers of SNPs (Alexander et al., 2005; Ringquist et al., 2005). The program accepts as input either gene locus names or SNP identification numbers. The output is a list of oligonucleotide primers used during pyrosequence-based typing to evaluate the inheritance patterns of SNP markers, thus allowing the collection of genetic data for analysis of their linkage to the disease phenotype.

SOP³ Program Output

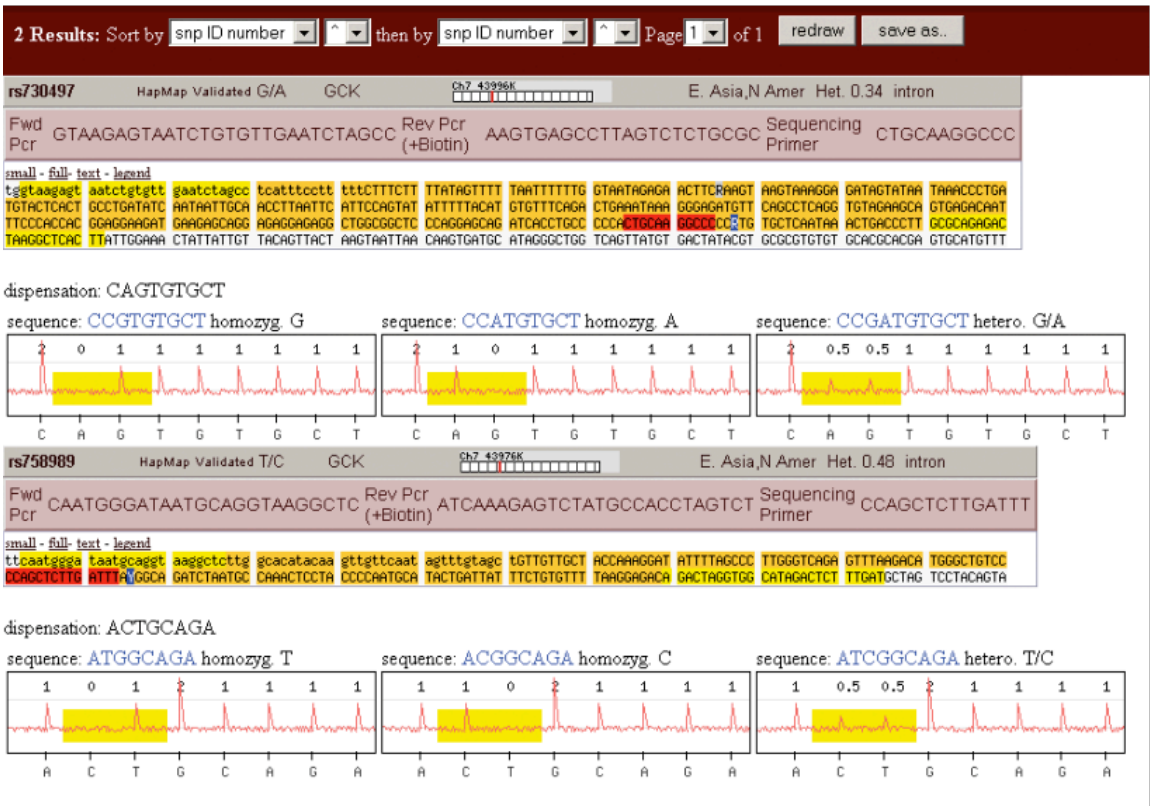

Figure 10. The output file obtained from the primer design software. The query specified that the application design pyrosequencing primers for all SNPs within the locus encoding glucokinase, GCK. Two primers set were returned with known validation in populations recruited from North American. Primers for PCR and pyrosequencing are indicated in each panel. The location of the SNP (blue) and the expected data that will be obtained are also indicated.

Design of locus specific primer sets for use during genetic analysis requires combining information from multiple sources, and as a result can be time consuming to develop when validating assays for large numbers of polymorphisms. Software was written for the development of genotyping assays (Alexander et al., 2005; Ringquist et al., 2005). The application uses warehoused data from existing genome projects along with the identity and location of relevant polymorphisms (i.e., SNPs as well as insertion/deletion events) for analysis by the application's primer design algorithm. The method automates processes such as collection of the genomic sequence, identification of polymorphic residues, and locus-associated functional information. It also takes into consideration restrictions that should be applied for sequencing applications, such as optimum length of PCR amplification product, minimum distance between sequencing primer and polymorphic residue, and the presence of nearby polymorphisms. The output is a list of oligonucleotide primers recommended for use in sequence-based genotyping to evaluate the inheritance patterns of SNP markers, enabling the collection of genetic data for analysis of their association to inherited phenotypes.

The Genotype View module enables both remote macro-level view with summary in the form of a progress bar, two unique closeup views of the assay trays, and a view of the sequencing data. The software uses the data from the genotyping file to draw a simulation of the genotyping data from the samples in the sequencing trays (Figure 11). As illustrated in the example, when a tray is expanded in size for a closeup view, sequencing successes "passed" (shown in blue), errors "failed" (shown in red), and marginal "check" results (shown in orange) are shown.

MAESTRA Viewer for Genotyping Data

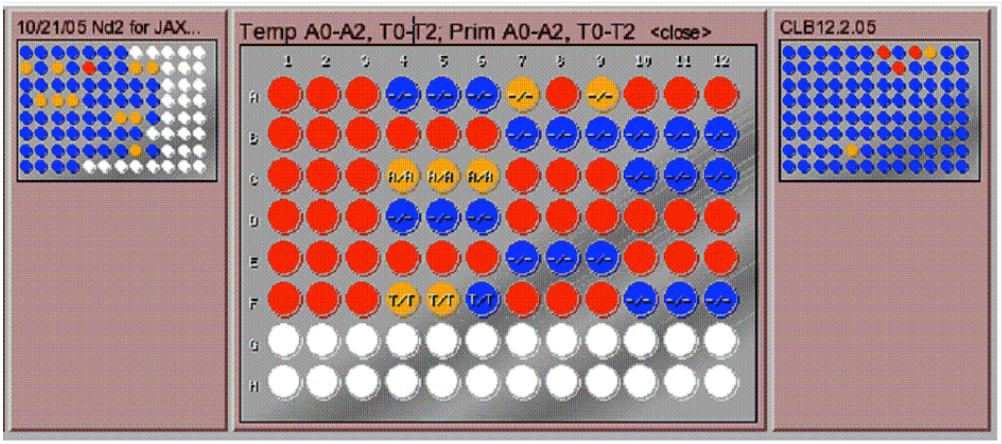

Figure 11. The Genotype View module enables custom views of the genotyping results. Macro-level views of multiple 96-well pyrosequencing trays are shown. When a tray is expanded in size for a closeup view (center panel), sequencing successes "passed" (shown in blue), errors "failed" (shown in red), and marginal "check" results (shown in orange) are highlighted.

The View Linkage module shows the linkage results for the candidate regions, producing an adjustable genome-wide "hot-spot" map, where the linkage score for each candidate variation, determined through the chosen genetic test, is shown as an color-coded ellipse (Figures 12, top panel). The color coding range follows a cold/hot scheme and is stated as follows: the blue colors indicate significant linkage (p-value<0.05) while the brighter red indicates highly-significant linkage (p-value<0.001). The locus names may be displayed if the SNP is contained within the boundaries of a gene. Also, linkage maps containing data from the International HapMap Consortium may produce linkage maps that surround the region of the SNPs (Figure 12, bottom panel).

Genetic Association Viewer

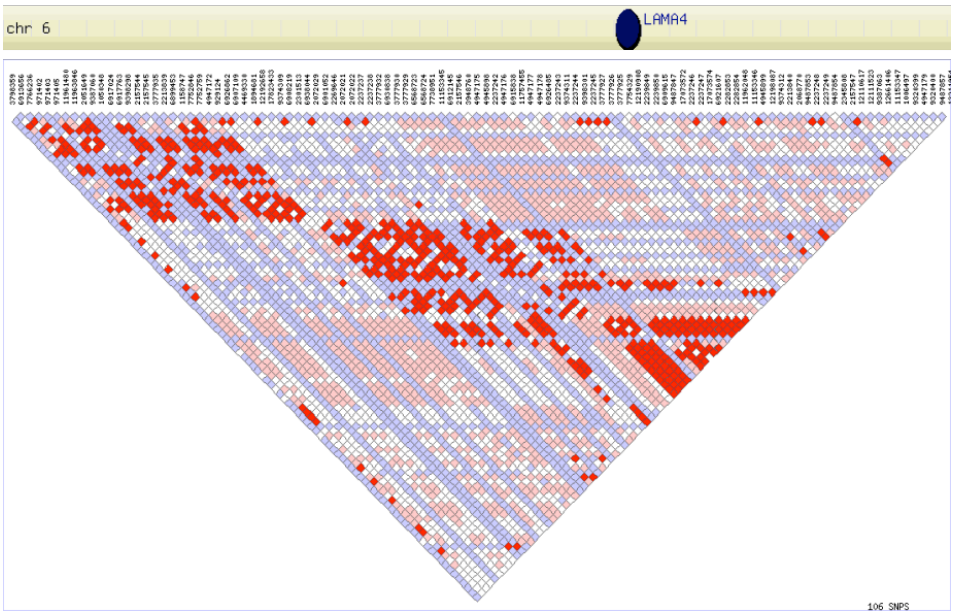

Figure 12. The View Linkage module illustrates the results from genotyping (Top Panel). The color coding range follows a cold/hot scheme and is stated as follows: blue colors indicate significant linkage (p-value<0.05) while the brighter red indicates highly-significant linkage (p-value<0.001). Locus names may be displayed if the SNP is contained within the boundaries of a gene. Linkage disequilibrium maps containing data from the International HapMap Consortium produce maps that surround the region of the SNPs (Bottom Panel).

Development of web-based software tools for analysis of complex genetic disease uses the web-based software applications our group has developed to design PCR and pyrosequencing primers optimized for identifying SNPs associated with enhanced risk for developing T1D and its complications. Our computational approach exploits genomic data deposited as part of the human genome project. The aim is to provide computational support for assay development, sample tracking, and data analysis for the T1D complications genotyping projects. Providing a web accessible application allows us to disseminate the results of assay design with other members of our research collaboration located at either end of the U.S. Software applications are being written and implemented in order to manage laboratory workflow, track sample inventory, develop genotyping assays, and automate the analysis of genotyping results (Figure 13). All products of the laboratory software suite are available on a web-based computational server located at URL: http://imgen.ccbb.pitt.edu.

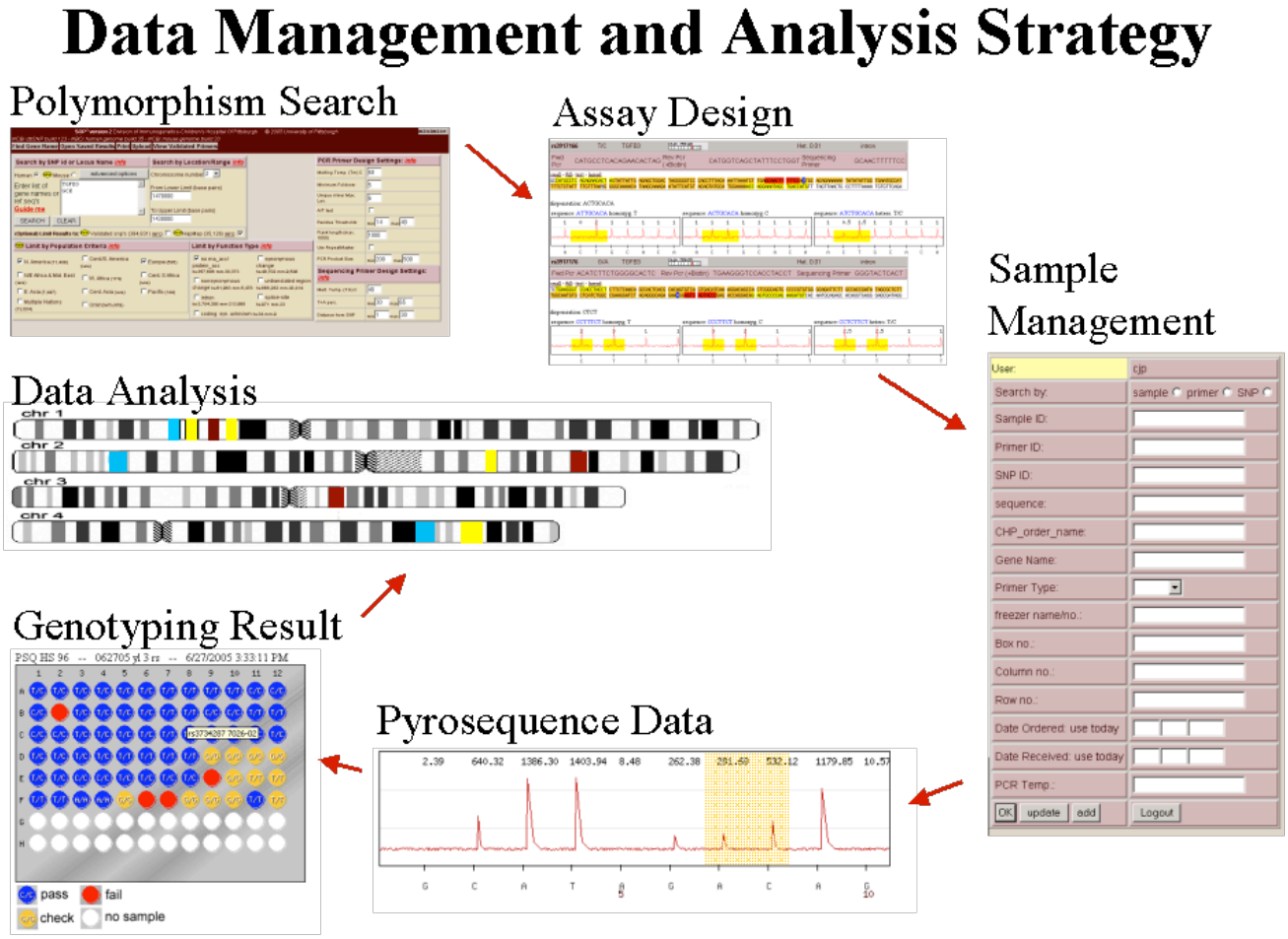

Figure 13. Strategy for developing a laboratory data management and analysis system for evaluation of the genetics of T1DN. The analysis system uses a web-based application along with an integrated database for sample management (Ringquist et al., 2005). Laboratory data obtained by pyrosequence-based typing will be used to calculate genomic hot spots (indicated in red) for linkage to the phenotype.

The glomerular filtration barrier. A thorough understanding of the molecular properties of the glomerular filtration barrier is crucial to the comprehension of the pathology of proteinuria encountered in diabetic nephropathy. Structurally the glomerular filtration barrier is composed of three layers which mediate the process of ultrafiltration of the blood flowing through anastomosing capillary loops leading to the formation of the primary urine in the Bowman`s capsule and finally into the tubular system. The first layer is a highly fenestrated vascular endothelium that covers the wall of the capillaries. The second layer is the glomerular basement membrane (GBM), forming the boundary between blood and urine primarily composed from an interconnecting network of type IV collagen (alpha-1 and -2 during fetal development and alpha-3, -4, and -5 in the adult) and laminin-1 (consisting of alpha-1/beta-1/gamma-1 chains in the fetus) and laminin-11 (consisting of alpha-5/beta-2/gamma-1 chains in the adult). The third layer is composed of highly specialized cells, the podocytes whose cytoplasm extends into interdigitating pedicles or foot processes. The area between the podocyte foot processes is the slit diaphragm composed of a number of cell-surface proteins including nephrin, podocalyxin, and P-

cadherin. The slit diaphragm formed by these proteins ensures that large macromolecules such as serum albumin and gamma globulin remain in the bloodstream while allowing small molecules such as water, glucose, and ionic salts to pass through (Pavenstadt et al., 2003).

The genesis of albuminuria is consistent with generalized vascular dysfunction leading to alterations in extracellular matrix components and impaired basement membrane organization resulting from hyperglycemia (Deckert et al., 1989). In particular increasing consideration has been given to the glomerular filtration barrier and its central role in the filtration of plasma proteins. High molecular weight tracer molecules have been used to investigate the molecular sieving properties of the glomerular filtration barrier. Ferritin has an estimated Stokes Radius (SR) of 61 Angstroms, and when injected into healthy rats failed to pass through the glomerular basement membrane (Farquhar et al., 1961). In puromycin aminonucleoside-induced nephrosis (PAN) models, which induce glomerular injury evidenced by flattening of podocyte foot processes, ferritin is only partially retained (Farquhar and Palade, 1961). Smaller proteins have also been used to examine the selective permeability of the GBM. For example, horseradish peroxidase (SR = 30 Ang) has been observed to pass through both the GBM and slit diaphragm while myeloperoxidase (SR = 36 Ang) and catalase (SR = 52 Ang) are GBM permeable but are captured by the slit diaphragm prior to entering the urine, consistent with the hypothesis that for large proteins GBM selectivity is the primary barrier separating the blood and urine space (Pavenstadt et al., 2003).

It has been suggested that thickening of GBM found in the diabetic nephropathy kidney underlie a structural alteration of the membrane and that dysregulation of type IV collagens are involved in onset of disease (Kefalides, 1981). The GBM is composed of a variety of proteins including type IV collagen, proteoglycans and laminin. The collagen molecule is composed of three chains able to form a network structure of triple helical molecules. The adult GBM is composed of alpha -3, -4, -5, and -6 chains. In Alport syndrome, a genetically determined disease characterized by heavy proteinuria, defects in the type IV collagen molecule are responsible for the principal clinical feature of the syndrome. In these patients the pathology is sustained by an abnormal basement membrane due to defects and/or absence of type IV collagens (Hudson et al., 1993; Miner and Sanes, 1996).

Genetic polymorphisms of extracellular matrix proteins as well as enzymes involved in their metabolism have been suggested as possible explanations for the variation in susceptibility to kidney disease observed in T1D patients (Deckert et al., 1989; Van Agtmael et al., 2005). Type IV collagens are a principal component of basement membranes (Kalluri, 2003). In the glomerular basement membrane COL4A1 and COL4A2 are expressed in the earliest stages of glomerular development (Miner and Sanes, 1994). In the adult glomerulus, the fetal expressed forms COL4A1 and COL4A2 are replaced by collagens encoded by the COL4A3, COL4A4, and COL4A5 loci, and absence of the adult forms of collagen have been associated with proteinuria due to Alport syndrome. Single nucleotide polymorphisms associated with fetal expressed collagen COL4A1 have also been linked with susceptibility to nephropathy. Glomerular basement membrane abnormalities characterized by irregularities in the parietal epithelium lining of Bowman's capsule have been observed in heterozygous mice containing a single nucleotide mutation in COL4A1 resulting in a Gly627W amino acid substitution in exon 26 (Van Agtmael et al., 2005) as well as mice harboring a G to A polymorphism in a COL4A1 encoded splice site resulting in loss of exon 40 from the mRNA transcript (Gould et al., 2005). Loss of COL4A1 exon 40 has been linked to nearly 100% prevalence of albuminuria in animals by the time they reach 2 years of age (Gould et al., 2006). In humans, some families with small vessel disease also exhibit increased albuminuria along with increased risk of hemorrhagic stroke (Gould et al., 2005; Gould et al., 2006). While deletion of COL4A1 is lethal, animals die by embryonic day 9.5 (Van Agtmael et al., 2005), heterozygous individuals may survive to adulthood carrying increased risk of microvascular complications as a result of altered basement membrane structure occurring during early development and organogenesis.

Candidate genes and their pathways. Candidate genes examined during transmission/disequilibrium testing (TDT) of complete family trios from which the offspring experienced T1DN have been reported recently by Ewens et al. (2005). Genes were chosen based on the results of previous studies associating various regions of the human genome to the phenotype of T1DN and have implicated proteins expressed within the glomerular filtration barrier. Extracellular matrix proteins encoded by the loci COL4A1, LAMA4, and LAMC1 comprise important components of glomerular basement membrane forming the blood-urine barrier in the kidney. Genetic variants linked to different alleles of the COL4A1 locus have been studied for 72 family trios. TDT statistical analysis supported the hypothesis for highly significant genetic linkage of COL4A1 (p-value 0.002) to the nephropathy phenotype along with additional linkage to the laminin encoding loci, LAMA4 (p-value 0.02) and LAMC1 (p-value 0.03).

Other loci whose gene products are associated with function of the glomerular basement membrane have been studied as well and include matrix metalloproteinase-9 (MMP9), alpha integrin (ITGA3), and nephrin (NPHS1) (Ewens et al., 2005). Analysis of the ITGA3 and NPHS1 loci indicated no linkage to T1DN but alleles of the MMP9 loci have

provided evidence for linkage, exhibiting a p-value 0.03. MMP9 activity has been implicated in glomerulosclerosis models of kidney disease and has been implicated in regulation of podocin a principal component of the glomerular slit diaphragm (Gerke et al., 2005). Additional candidate genes implicated by their role in glomerular filtration are those expressed by the podocytes and comprise the slit diaphragm. The podocyte expressed proteins podocin (NPHS2), CD2-associated protein (CD2AP), p-cadherin (CDH3), and homolog of FAT tumor suppressor (FAT) proteins are principal components of the slit diaphragm. Genotyping of SNP markers in order to complete the analysis of the complete set of glomerular basement membrane proteins, and the integrated glomerular slit diaphragm, will realize the goal to assess the possible role of these loci in genetic risk to T1DN.

Gene products that regulate NADPH oxidase activity have been included in candidate gene approaches investigating the genetic risk associated with T1DN. The kidney expressed form of NADPH oxidase is produced in the kidney cortex and in podocytes (Pavenstadt et al., 2003). The catalytic activity of NADPH oxidase utilizes reducing equivalents from NADPH to convert oxygen molecule into super oxide radical. Increased activity of the enzyme has been correlated with nephropathy in animal models (Neale et al., 1993; Kerjaschki and Neale, 1996). Conditions that down regulate the activity of NADPH oxidase contribute positively to improved renal function. Partial genetic dissection of the human expressed form of NADPH oxidase has been accomplished by TDT analysis of family trios. As reported by Ewens et al. (2005), analysis of the transmission data obtained from family trios have implicated the gene product of the CYBA locus, encoding the p22phox subunit, as being correlated with T1DN and exhibiting a p-value of 0.03. Other subunits of NADPH oxidase were also investigated. Analysis of the catalytic subunit NOX4 in the same set of family trios has thus far exhibited no association to the phenotype.

A protein that increases the activity of NADPH oxidase is protein kinase C beta 1 subunit (PRKCB1) while the product of nitric oxide synthase 3 (NOS3) inhibits enzyme activity. The subunit beta-1 of protein kinase C (PRKCB1) has been examined among the cohort of 72 family trios and exhibited a statistically significant linkage to the phenotype, presenting a p-value of 0.05 (Ewens et al., 2005). The enzymatic action of protein kinase C has been associated with increased enzymatic activity in patients exhibiting nephropathy (Araki et al., 2003). The gene products glutathione peroxidase (GPX1) and catalase (CAT) are components of the super oxide radical scavenging system present in the kidney cortex. Analysis by the transmission disequilibrium test of loci GPX1 and CAT provided evidence for linkage to T1DN with p-values of 0.02 and 0.04, respectively (Ewens et al., 2005).

The evidence for a dominant genetic role in determining susceptibility to kidney disease in T1D patients is primarily the result of epidemiological studies indicating that prevalence of DN increased during the first 15 years after onset of T1D. After 20 years duration of diabetes the incidence of new cases of nephropathy among T1D patients plateaus and may in fact decrease. These observations of T1D populations have frequently been interpreted as indicating that there exists a subset of patients susceptible to development of kidney disease. Additional evidence for genetic risk has been obtained from family studies showing the clustering of DN among T1D siblings. Siblings experiencing T1D have a significantly increased risk for DN when the T1D proband experiences the disease. Interpretation of the genetic analysis of T1DN is complicated by the possibility that signals are related to coincident diseases, especially T1D. In order to interpret these results it is necessary to compare the results of genetic testing of control populations as well as replication of the results in independently recruited case cohorts. In the example of candidate genes, choosing loci for their potential role in kidney disease separate from loci associated with T1D may additionally aid the resolution of genetic influence on the syndrome.

Principal conclusions. Although the results described above from our screen of candidate genes exceeded the threshold of statistical significance (i.e., $p<0.05$), they must be considered preliminary in view of the small sample size (i.e., 92 family trios). In order to confirm and expand these results, additional families are being collected in Pittsburgh, Hawaii and at Walter Reed as well as through our collaboration with the Juvenile Diabetes Research Foundation. In the regions of suggestive associations, the research will continue to test additional SNPs identified from the Celera database and public databases as well as by sequencing of interesting candidate regions.

The transmission/disequilibrium test (TDT) has been used to analyze genetic polymorphisms within 121 candidate genes for association with type 1 diabetic-nephropathy (T1DN) in 92 family trios, including 88 parent-child trios and 4 multiplex families. The proband in all families had T1D and either end-stage renal disease or proteinuria with urinary albumin/creatinine ratio >300 ug/mg. Results of TDT were significant for 13 loci (p-value < 0.05) and highly significant for 4 loci (p-value < 0.005), while the remaining 108 loci have exhibited no evidence of genetic association with the disease. TDT and related methods can detect the effect of genes that make a relatively small contribution, provided that susceptibility alleles are in linkage disequilibrium with marker alleles. Genetic risk of developing T1DN has been observed to increase almost 3-fold in siblings of persons with T1DN and exhibit prevalence for the disease of greater than 70%. Inheritance of increased risk for developing T1DN is consistent with a dominant mode of inheritance. Simulations of

dominant inheritance models designed to identify allelic polymorphisms linked to the disease marker, and present between 10% and 80% in the study population, indicate that between 150 and 300 family trios would need to be genotyped to achieve 80% power when applying the TDT.

KEY RESEARCH ACCOMPLISHMENTS:

Research Accomplishments Emanating from the Research Project:

Greater than 630 Family trios (325 cases and 312 controls) and 1,174 singletons (578 cases and 596 controls) have been obtained to allow identification of genetic markers linked to diabetic complications, including diabetic-nephropathy.

Development, testing, and publication of software applications for design of genotyping assays have been completed and are used to optimize assays for genetic analysis.

Allele transmission data curently identify 4 loci with highly significant association with diabetic nephropathy.

Nominally significant results have been found with SNPs in several genes that were tested:
BCL2: SNP rs2062011 in intron 1 of BCL2 exceed a p-value of 0.003 for association with diabetic nephropathy.

COL4A1: The SNP rs679062 in intron 1 of COL4A1 has significant TDT results, p-value less than 0.003.

LPL: Two SNPs rs326, and rs320 (located in intron 8) and SNP rs13702 (3' untranslated region) are associated with p-values exceeding 0.005.

SMAD3: Analysis of SNP rs12594610 in intron 1 has provided evidence associating the SMAD3 locus with end-stage renal disease. The p-values exceeded 0.005.

Although the results shown above are nominally significant, they must be considered preliminary in view of the small sample size. In order to confirm and expand these results, additional families are being collected by Dr. Trucco and additional SNPs in the regions of suggestive associations (identified from the Celera database and public databases such as NCBI SNPdb, as well as by sequencing of interesting candidate regions) are being tested in all available families.

REPORTABLE OUTCOMES:

Dr. Trucco's laboratory has published 10 manuscripts in 2006 and 71 manuscripts in peer reviewed journals during the funding period 2001 to present (selected publications are listed below):

Pasquali L, Fan Y, Trucco M, Ringquist S. Rehabilitation of adaptive immunity and regeneration of beta cells. **Trends Biotechnol.** 24:516-522, 2006.

Balamurugan AN, Chang Y, Bertera S, Sands A, Shankar V, Trucco M, Bottino R. Suitability of human juvenile pancreatic islets for clinical use. **Diabetologia** 49:1845-1854, 2006.

Wang Z, Zhu T, Rehman KK, Bertera S, Zhang J, Chen C, Papworth G, Watkins S, Trucco M, Robbins PD, Li J, Xiao X. Widespread and stable pancreatic gene transfer by adeno-associated virus vectors via different routes. **Diabetes** 55:875-884, 2006.

Harnaha J, Machen J, Wright M, Lakomy R, Styche A, Trucco M, Makaroun S, Giannoukakis N. Interleukin-7 is a survival factor for CD4+ CD25+ T-cells and is expressed by diabetes-suppressive dendritic cells. **Diabetes** 55:158-170, 2006.

Rood PP, Bottino R, Balamurugan AN, Fan Y, Cooper DK, Trucco M. Facilitating physiologic self-regeneration: a step beyond islet cell replacement. **Pharm Res.** 23:227-242, 2006.

Bottino R, Trucco M. Multifaceted therapeutic approaches for a multigenic disease. **Diabetes** 54:S79-S86, 2005.

Casu A, Trucco M, Pietropaolo M. A look to the future: prediction, prevention, and cure including islet transplantation and stem cell therapy. **Pediatr Clin North Am.** 52:1779-1804, 2006.

Ringquist S and Trucco M. Regenerative medicine for diabetes treatment. **Discovery Medicine** 5:142-147, 2005.

Ringquist S, Pecoraro C, Gilchrist CM, Styche A, Rudert WA, Benos PV, Trucco M. SOP3v2: web-based selection of oligonucleotide primer trios for genotyping of human and mouse polymorphisms. **Nucleic Acids Research** 33:W548-552, 2005.

Trucco M. Regeneration of the pancreatic beta cell. **Journal Clinical Investigation** 115:5-12, 2005.

Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, Ball S, Specht SM, Polejaeva IA, Monahan JA, Jobst PM, Sharma SB, Lamborn AE, Garst AS, Moore M, Demetris AJ, Rudert WA, Bottino R, Bertera S, Trucco M, Starzl TE, Dai Y, Ayares DL. Production of alpha 1,3-galactosyltransferase-deficient pigs. **Science** 299:411-414, 2003.

Friday RP, Profozich J, Pietropaolo S, Trucco M, Pietropaolo M: Alternative core promoters regulate tissue-specific transcription from the autoimmune diabetes-related *ICA1* (ICA69) gene locus. **Journal of Biological Chemistry** 278:853, 2003.

Bottino R, Lemarchand P, Trucco M, Giannoukakis N: Gene and cell-based therapeutics for type 1 diabetes mellitus. **Gene Therapy** 10:875, 2003.

Dorman JS, Charron-Prochownik D, Siminerio L, Ryan C, Poole C, Becker D, Trucco M: Need for genetic education for type 1 diabetes (letter). **Archives of Pediatric Adolescent Medicine** 157:935, 2003.

Ringquist S, Alexander AM, Styche A, Pecoraro C, Rudert WA, Trucco M: HLA class II DRB high resolution genotyping by pyrosequencing: comparison of group specific PCR and pyrosequencing primers. **Human Immunology** 65:163, 2004.

Barinas-Mitchell E, Pietropaolo S, Zhang YJ, Henderson T, Trucco M , Kuller LH, Pietropaolo M: Islet cell autoimmunity in a triethnic adult population of the Third National Health and Nutrition Examination Surveys (NHANES) III. **Diabetes** 53:1181, 2004.

Alexander AM, Pecoraro C, Styche A, Rudert WA, Benos PA, Ringquist S, Trucco M: SOP^3: A web-based tool for selection of oligonucelotide primers for single nucleotide polymorphism analysis. **BioTechniques** 38:87-94, 2005.

Dr. Trucco's laboratory has published 4 invited book chapters during 2006 (publications are listed below):

Ringquist S, Nichol L, and Trucco M. Transplantation genetics. *In* Rimoin D, Connor JM, Pyeritz R, Korf B. Emery A (eds): *Emery and Rimoin's Principles and Practice of Medical Genetics 5th Edition*. Churchill Livingstone (2006).

Ringquist S, Styche A, Rudert WA, and Trucco M. Pyrosequence-based strategies for improved allele typing of HLA loci. *Methods in Molecular Biology* (2006).

Ringquist S, Pecoraro C, Lu Y, Styche A, Rudert WA, and Trucco M. Web-based primer design software for genome scale SNP mapping by pyrosequencing. *Methods in Molecular Biology* (2006).

Ringquist S and Trucco M. Regenerating pancreatic islet function in juvenile diabetes. *Progress in Stem Cell Research*. Nova Science Publishers (2007).

Dr. Trucco's laboratory has presented 10 invited lectures and meeting abstract in 2004-2006 (publications are listed below):

Ringquist S and Trucco M. A proteomics approach necessary to confirm molecularly determined associations between genes and nephropathy. Department of Defense Symposium on Advanced Technology to Improve Prediction and Prevention of Type 1 Diabetes Mellitus, 2004.

Ringquist S, Ge X, Zhang L, Styche L, Balamurugan AN, Bottino R, Rudert WA, Trucco M. Proteomic scanning for markers associated with successful islet isolation and maintenance. Keystone Symposia Diabetes Mellitus, 2004.

Ringquist S, Rudert WA, and Trucco M. New advanced technology to improve prediction and prevention of type 1

diabetes. Department of Defense Symposium on Telemedicine and Advanced Technology Research Center, 2004.

Pecoraro C, Styche A, Rudert WA, Benos PV, Ringquist S, Trucco M. SOP^3: A web based tool for selection of oligonucleotide primers for SNP analysis. University of Pittsburgh Science 2004 Symposia, 2004.

Ringquist S, Pecoraro C, Styche A, Lu Y, Rudert WA, and Trucco M. Genetic analysis of complex disease: web-base management tool for DNA polymorphisms. Windber Research Institute Showcase for Biotechnology, 2005.

Ringquist S and Trucco M. Genetic analysis of complex disease. Windber Research Institute Showcase for Biotechnology, 2005.

Ringquist S, Pecoraro C, Styche A, Lu Y, Rudert WA, and Trucco M. Genetic analysis of complex disease: web-base management tool for DNA polymorphisms. University of Pittsburgh Symposium Science, 2005.

Ringquist S and Trucco M. SOP^3: web-based selection of oligonucleotide primer trios for genotyping of human and mouse polymorphisms. University of Pittsburgh Symposium Science, 2005.

Trucco M. New Advanced Technology to Improve Prediction and Prevention of Type 1 Diabetes. Department of Defense Symposia on Distance Medicine, Fredrick, MD (Jan. 2006).

Pecoraro C, Lu Y, Pasquali L, Styche A, Rudert WA, Trucco M and Ringquist S. MAESTRA software in genome-wide and candidate gene analysis: a case study. Windber Research Institute Symposium Translational Health, August 13-15, 2006.

Patents and Licenses (applied for and/or issued)

Copyrighted Material:
SOP3 software for designing primers for PCR and pyrosequencing.

Degrees Obtained that are Supported by this Award

N/A

Development of Cell Lines

Lymphoblastoid cell lines from the Children's Hospital of Pittsburgh collection of diabetic families.

Tissue or Serum Repositories

Repository of almost 2,881 DNA samples from family trios and singletons in which the proband exhibited T1D, T1DN, or T1D-ESRD have been obtained.

Informatics and Databases

Development, testing, and publication of software application for designing assays for genotyping of human DNA samples.

Warehousing of databases of the human genome, human genetic polymorphisms, and human haplotype maps.

Animal Models
None

Funding Applied for Based on Work Supported by this Award
None

Employment or Research Opportunities Applied for and/or Received Based on this Award
None

CONCLUSIONS:

Summary of the Importance and/or Implications of the Research

Healthcare costs associated with diabetes and diabetic complications, such as nephropathy, account for 10% of monies spent on healthcare. Early identification of individuals at risk for this chronic disease will aid in improved management, decreased severity, and reduced healthcare costs. Potential benefits of the project are: (1) improved forecasting of the genetic risk of developing diabetes and diabetic complications; and (2) the opportunity to apply preventative treatment focused on at-risk individuals.

Recommended Changes on Future Work

Challenges to successful completion of the project are as follows:

1. Obtaining the quantity of samples to be analyzed in order to provide statistically significant results.

We have arranged through our association with the Children's Hospital of Pittsburgh, the University of Pittsburgh School of Medicine Transplantation Institute, the University of Hawaii, and the Walter Reed Hospital in Washington, DC, and the Juvenile Diabetes Research Foundation to receive genomic DNA samples from at least 300 T1DN family trios (father, mother, and diabetic offspring) in order to evaluate a sufficiently large number of allelic transmissions for statistically significant genotyping analysis. A total of 581 T1D family trios have also be obtained. These samples consist of genomic DNA as well as health records to allow evaluation of the presence of diabetic complications. Family trios of diabetic complications for retinopathy and neuropathy are still to be collected.

2. The need to validate alternative methods for monitoring the accuracy of the genotyping data.

During the course of the project we have insured the accuracy of our genotyping approach by obtaining data on duplicate samples. We will also introduce in the next research period an alternative method for genotyping by microarray analysis. This will be preformed using a human polymorphism microarray manufactured by Affymetrix Corporation. The throughput of this system will allow, in a single run, genotyping of 500,000 loci from 197 case (type 1 diabetes and end-stage renal disease) and 197 controls (type 1 diabetes but with healthy kidney function), providing confirmation of the pyrosequence-based genotyping protocol as well as extending our analysis to other genomic loci.

3. Development and testing of software for data analysis as well as inventory management.

Development of laboratory data management software and custom databases for the warehousing of genomic information has been pursued. We are currently focused on finishing a lab management system for sample tracking and data analysis.

Military Relevance of the Research Project:

Diabetes affects 16 million Americans (greater than 5% of the population), and 800,000 new cases annually.

Diabetes occurs in men, women, children and the elderly. African, Hispanic, Native and Asian Americans are particularly susceptible to its most severe complications.

Diabetic kidney disease accounts for 42% of new cases of end-stage renal disease, with over 100,000 cases per year at an average cost of $55,000 per patient annually.

Economic impact of diabetes is over $100 billion annually accounting for more than $1 in every $10 healthcare dollars and $1 of every $4 Medicare dollars spent.

As the military is a reflection of the US population, improved prediction of risk for developing diabetes and diabetic complications amongst active duty members of the military, their families and retired military personnel will potentially allow focused preventative treatment of at-risk individuals, providing significant healthcare savings and improved patient well being.

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APPENDIX A (modified 05/03/06 SR)
GENETIC STUDIES OF DIABETIC COMPLICATIONS
(Survey for Family Members of Index Case)

Study Title: New Advanced Technology to Improve Prediction and Prevention of Type I Diabetes

Name_____ Date of Contact_____

Address_____ Date of Birth_____

City_____ State_____ Zip Code_____

Phone_____ Race (AA, C, H)_____ Index Case_____

Relation to Index (Parent, Sib, Offspring, Spouse, etc)_____

Willingness to participate: ☐ Yes ☐ No

General Information and Health History:

Birth Weight:_____Current Weight:_____Current Height:_____Age at Puberty:_____Gender:_____

Hypertension (High Blood Pressure): ☐ Yes ☐ No If yes, duration_____

Are you currently a smoker? ☐ Yes ☐ No If yes, duration_____

Have you previously been a smoker? ☐ Yes ☐ No If yes, duration_____

May we contact you for more information? ☐ Yes ☐ No Best time of day to call_____

Health History at Onset of Diabetes: (<u>If you have diabetes please answer the following questions.</u>)

Do you have diabetes? ☐ Yes ☐ No If yes please specify: ☐ Type 1 ☐ Type 2 ☐ Type Unknown

Duration of Diabetes:_____Age at Onset of Diabetes:_____Weight at Onset of Diabetes:_____

Symptoms Leading to Diagnosis of Diabetes:

☐ Loss of weight ☐ Frequent urination ☐ Excessive thirst ☐ Bad breath ☐ Autoimmune disease

☐ Asymptomatic ☐ Casual discovery by Physician ☐ Other, please specify_____

Diabetic Complications:

Nephropathy (Damage to Kidneys): ☐ Yes ☐ No If yes, duration_____

Retinopathy (Damage to Eyes): ☐ Yes ☐ No If yes, duration_____

Neuropathy (Nerve and Joint Pain): ☐ Yes ☐ No If yes, duration_____

Medications and Therapies for Treating Diabetes and Diabetic Complications:

Therapy at diagnosis of diabetes: ☐ None ☐ Diet ☐ Insulin

☐ Oral Meds, please specify_____ ☐ Other, please specify_____

Current therapy for diabetes: ☐ None ☐ Diet ☐ Insulin

☐ Oral Meds, please specify_____ ☐ Other, please specify_____

What is your typical HbA_{1c} level? ☐ < 6.0% ☐ 6.0% to 7.2% ☐ 7.2% to 8.4% ☐ > 8.4%

Medication for kidney problems or hypertension:_____

Are you receiving dialysis for kidney problems? ☐ Yes ☐ No Have you had a kidney transplant? ☐ Yes ☐ No

Related Medical Concerns: ☐ Thyroiditis ☐ Celiac Disease ☐ Other_____

APPENDIX B (modified 05/03/06 SR)

GENETIC STUDIES OF DIABETIC COMPLICATIONS

(Index Case Family Information)

Study Title: New Advanced Technology to Improve Prediction and Prevention of Type I Diabetes

Name:_____ Date of Birth:_____

Address:_____ INDEX CASE:_____

City:_____State:_____ Zip Code:_____

Phone Number (Home)(_____)_____ Phone Number (Work)(_____)_____

General Health History:

Birth Weight:_____Current Weight:_____Current Height:_____Age at Puberty:_____Gender:_____

Health History at Onset of Diabetes:

Do you have diabetes? ☐ Yes ☐ No If yes please specify: ☐ Type 1 ☐ Type 2 ☐ Type Unknown

Duration of Diabetes:_____Age at Onset of Diabetes:_____Weight at Onset of Diabetes:_____

Symptoms Leading to Diagnosis of Diabetes:

☐ Loss of weight ☐ Frequent urination ☐ Excessive thirst ☐ Bad breath ☐ Autoimmune disease

☐ Asymptomatic ☐ Casual discovery by Physician ☐ Other, please specify_____

Diabetic Complications:

Nephropathy (Damage to Kidneys): ☐ Yes ☐ No If yes, duration_____

Retinopathy (Damage to Eyes): ☐ Yes ☐ No If yes, duration_____

Neuropathy (Nerve and Joint Pain): ☐ Yes ☐ No If yes, duration_____

Hypertension (High Blood Pressure): ☐ Yes ☐ No If yes, duration_____

Medications and Therapies for Treating Diabetes and Diabetic Complications:

Therapy at diagnosis of diabetes: ☐ None ☐ Diet ☐ Insulin

☐ Oral Meds, please specify_____ ☐ Other, please specify_____

Current therapy for diabetes: ☐ None ☐ Diet ☐ Insulin

☐ Oral Meds, please specify_____ ☐ Other, please specify_____

What is your typical HbA_{1c} level? ☐ < 6.0% ☐ 6.0% to 7.2% ☐ 7.2% to 8.4% ☐ > 8.4%

Medication for kidney problems or hypertension:_____

Are you receiving dialysis for kidney problems? ☐ Yes ☐ No Have you had a kidney transplant? ☐ Yes ☐ No

Related Medical Concerns: ☐ Thyroiditis ☐ Celiac Disease ☐ Other_____

Are you currently a smoker? ☐ Yes ☐ No If yes, duration_____

Have you previously been a smoker? ☐ Yes ☐ No If yes, duration_____

Please circle race: (**optional**) African American, Asian, Caucasian, Hispanic, Other_____

May we contact you for more information? ☐ Yes ☐ No Best time of day to call_____

INDEX CASE:____________________

FAMILY INFORMATION

PARENTS

Father: Age__________ Living: ▯ Yes ▯ No

Does your father have diabetes? ▯ Yes ▯ No If yes, Duration of diabetes___________Treatment___________

If your father has diabetes does he have: ▯ Type I ▯ Type 2 ▯ Type Unknown

Mother: Age__________ Living: ▯ Yes ▯ No

Does your mother have diabetes? ▯ Yes ▯ No If yes, Duration of diabetes___________Treatment___________

If your mother has diabetes does she have: ▯ Type I ▯ Type 2 ▯ Type Unknown

SIBLINGS:

Please list all brothers and sisters and whether they have diabetes:

Brother/Sister Age__________ Duration of diabetes__________ Living: ▯ Yes ▯ No

If the sibling has diabetes do they have: ▯ Type I ▯ Type 2 ▯ Type Unknown

Brother/Sister Age__________ Duration of diabetes__________ Living: ▯ Yes ▯ No

If the sibling has diabetes do they have: ▯ Type I ▯ Type 2 ▯ Type Unknown

Brother/Sister Age__________ Duration of diabetes__________ Living: ▯ Yes ▯ No

If the sibling has diabetes do they have: ▯ Type I ▯ Type 2 ▯ Type Unknown

Brother/Sister Age__________ Duration of diabetes__________ Living: ▯ Yes ▯ No

If the sibling has diabetes do they have: ▯ Type I ▯ Type 2 ▯ Type Unknown

Brother/Sister Age__________ Duration of diabetes__________ Living: ▯ Yes ▯ No

If the sibling has diabetes do they have: ▯ Type I ▯ Type 2 ▯ Type Unknown

Please list your children and whether they have diabetes:

Daughter/Son Age__________ Duration of diabetes__________ Living: ▯ Yes ▯ No

If the child has diabetes do they have: ▯ Type I ▯ Type 2 ▯ Type Unknown

Daughter/Son Age__________ Duration of diabetes__________ Living: ▯ Yes ▯ No

If the child has diabetes do they have: ▯ Type I ▯ Type 2 ▯ Type Unknown

Daughter/Son Age__________ Duration of diabetes__________ Living: ▯ Yes ▯ No

If the child has diabetes do they have: ▯ Type I ▯ Type 2 ▯ Type Unknown

Daughter/Son Age__________ Duration of diabetes__________ Living: ▯ Yes ▯ No

If the child has diabetes do they have: ▯ Type I ▯ Type 2 ▯ Type Unknown

Daughter/Son Age__________ Duration of diabetes__________ Living: ▯ Yes ▯ No

If the child has diabetes do they have: ▯ Type I ▯ Type 2 ▯ Type Unknown

Are there other relatives with diabetes, including Uncles, Aunts, Nephews, Nieces, Cousins, etc:

____

____

Appendix B, page 2

Genetic Screening in Diabetes
Walter Read Health Care System
Robert A. Vigersky, M.D., COL MC

INTRODUCTION:
The hypothesis to be tested is that there are allelic variations of some genes that make the development of diabetes-related complications more likely in patients who carry them than those who do not. The 3 major complications to be evaluated are diabetic nephropathy, diabetic neuropathy, and diabetic retinopathy. This is an observational study in which we will obtain DNA samples from the blood of patients with one or more of these complications and from as many their first-degree relatives as possible for testing in the laboratory of Dr. Massimo Trucco. He will evaluate these samples by studying candidate genes selected *a priori* and testing for transmission/disequilibrium – a standard for analysis of linkage between a candidate gene and a specific disease. This study extends the scope of the initially proposed study of nephropathy related genes by including patients and relatives who have neuropathy and/or retinopathy. The specific aims of the study are:
Aim 1. Obtain blood for genetic analysis from patients with diabetes mellitus complicated by nephropathy, autonomic neuropathy, or retinopathy and from their parents and/or siblings.
Aim 2. Determine whether any or all of these complications are linked to one of more of the proposed candidate genes.

BODY:
D. DESCRIPTION OF WORK.

D1. The Title of this study is "Genetic Screening in Diabetes" This is an observational study in which the research team at WRAMC will obtain DNA samples from the blood of patients with one or more diabetic complications (as specified in the protocol) and from as many of their first-degree relatives as possible for genetic testing. The Study will be performed at WRAMC for DEERS-eligible patients and at Uniformed Services University of Health Sciences (USUHS) for non- DEERS eligible patients.

D.2. After meeting eligibility parameters, a medical history interview will be conducted on all participants. Additionally, a quality of life questionnaire (QOL) will be administered. All participants will undergo a physical examination and an electrocardiogram. Participants will be scheduled for blood and urine sampling and analysis. If the participant's parents and/or sibling have diabetes, records will be requested from their primary care provider or endocrinologist for review of the status of their diabetes. A urine specimen, an Oral Glucose Tolerance Test and fingerstick capillary blood samples will be obtained on these individuals.

D.3. All samples will be typed and examined to evaluate if there are reasonable candidates that contribute to genetic susceptibility to diabetic nephropathy, retinopathy, and nephropathy. The initial emphasis will examine candidate gene analysis in families for nephropathy, autonomic neuropathy, and retinopathy. It is expected that WRAMC will enroll up to 100 participants and 300 of their family members.

D.5 All performance under this SOW will cease at either the completion of all work, unilateral or mutual termination, or October 31, 2008, whichever occurs first.

The strategy for starting this study at WRAMC was to use the protocol generated by Drs. Burgess and Birkmire-Peters once approved by the IRB at the University of Hawaii and by the Human Subjects Research Review Board (HSRRB) of USAMRMC. These approvals were not completed until April 2005.

We began WRAMC protocol submission process shortly thereafter but a major issue arose in discussions prior to the submission to the WRAMC IRB. We were informed by the IRB that the relatives of the probands who were not military health care beneficiaries could not be evaluated at WRAMC. We then sought an alternative site at the Uniformed Services University of the Health Sciences (USUHS) where we would establish a complete on-site facility to perform autonomic neuropathy testing, non-mydriatic retinal photography, and the

other studies required by the protocol. However, after initial discussions with our USUHS colleagues (Appendix 1) led us to believe that we would be able to use their facility on the campus of the National Naval Medical Center (NNMC) in Bethesda, Maryland, we were ultimately denied access to it despite their initial assurances (Appendix 2). We now have obtained alternate space at an office in Rockville, Maryland which will be sub-leased from USUHS investigators (Appendix 3) . Furthermore, USUHS required their own IRB approval and further required that a billeted faculty member must be the Principal Investigator (PI) on the submitted protocol. Therefore, they initially rejected my submission on procedural grounds (Appendix 4). Dr. Kevin Leary has agreed to be the PI for the USUHS part of the project (Appendix 5).

To date, the the following approvals have been obtained:
A) the Human Use Committee (the IRB at WRAMC) approved the protocol on 28 March 2006 after the establishment of a Memorandum of Agreement (MOU) between the WRAMC and Children's Hospital of Pittsburgh. The reasons for the MOU are: 1) to ensure that the non-WRAMC tissue bank itself has adequate quality controls, and to make sure WRAMC investigators have priority access to the tissue; and 2) to ensures that no tissue will be transferred for a secondary or tertiary use unless it is approved by the HUC. Especially, if secondary or tertiary use is for a non-WRAMC investigator, no tissue may be released unless it is under the provisions of an MOU. MOU's are required for the purpose to protect the ownership, use of the specimen, and the confidentiality of the specimen when WRAMC tissue is used and stored at non-WRAMC institution.
B) the Clinical Investigation Regulatory Office (CIRO) of the Department of the Army approved the protocol on 5 April 2006
C) the Cooperative Research and Development Agreement (CRDA) between the T.R.U.E. Foundation and the Department of Defense/United States Army Medical Research Acquisition Activity on 10 April 2006
D) the USUHS IRB gave tentative approval (pending revisions) on 12 October 2006 (Appendix 4). A subcommittee of the IRB will finalize the approval. The revisions are in the process of being completed.

KEY RESEARCH ACCOMPLISHMENTS:
The approval process has taken 18 months to date:

- HSRRB Approval – April 2005
- WRAMC HUC Approval - March 2006
- CIRO Approval – April 2006
- USUHS IRB Approval – October 2006

In view of the above, we have not begun any evaluations for this study. However, we have collected the names of 20 probands at WRAMC who have volunteered to be evaluated for inclusion in the study.

REPORTABLE OUTCOMES:
Approval of the protocol by all relevant IRB's.

CONCLUSIONS:
The arduous and circuitous approval process has been completed and patient recruitment is about to be begun.

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Risch N, Merikangas K: The future of genetic studies of complex human diseases. Science 273:1516-1517, 1996.

Risch N and Merikangas K. Genetic analysis of complex disease. Science 1997 275: 1329-1330.

APPENDICES:

Appendix 1

>>> "Vigersky, Robert A COL WRAMC-Wash DC"
>>> <Robert.Vigersky@na.amedd.army.mil> 10/12/05 11:11 AM >>>
Dear Dr. Cantilena,

It was a pleasure speaking with you this morning. As we discussed, the facilities that USUHS sublets in Building 141 on the campus of NNMC would be perfect for us. It is my understanding that individuals who are not military health care eligible are able to be seen and studied in that setting. I will have my Program Manager, Susan Walker, RN, contact your nurse Marion Kaiser about getting a tour. If you could just confirm your support in a return e-mail, I will forward that to DCI at WRAMC. Once the protocol is approved here, I will submit it to the USUHS IRB. Thanks much.

Wed 10/12/2005 11:46 AM

Dear Dr. Vigersky,

Likewise, it was a pleasure to speak with you as well. This is to confirm my support for your project and make available space in our outpatient research area for your study 1 or 2 half days per week per our discussion. It is my understanding is that you will provide your own research staff as well as administrative support for the project at this site. Please let me know if you require any further information to assist your research efforts.

Sincerely,

Lou Cantilena MD, PhD
Professor, Medicine and Pharmacology
Director, Division of Clinical Pharmacology and Medical Toxicology Department of Medicine
USUHS

Appendix 2

From: Kevin Leary [mailto:kleary@usuhs.mil]
Sent: Friday, September 01, 2006 9:32 AM
To: Vigersky, Robert A COL WRAMC-Wash DC; Roy, Michael J LTC (P) WRAMC-Wash DC
Cc: MROY@usuhs.mil; 'Goldstein, Robert'; Walker, Susan M Ms TRUE
Subject: RE: SPACE FOR MY GENETICS STUDY

Dr. Vigersky,

I again approached the NMRC representatives that handle clinical trials in bldg 141 concerning using that space for your genetics study. Although expressing a desire to collaborate, they feel as if they could not accommodate your needs for this particular study given there current protocols that are just getting initiated. In addition, the status of using bldg 141 for future research studies is still being clarified between NMRC and the host command, NNMC. Dr. Cantilena suggested utilizing some space off campus in White Flint being leased by the post polio syndrome group. There would be a cost associated with this but if you are interested let me know and I will look into this option. I do not think there would be any problem with me being the USU PI for this study but would need to get clearance through my department.

Regards,

Kevin

Kevin Leary, MD
MAJ, MC

Appendix 3

From: Kevin Leary [mailto:kleary@usuhs.mil]
Sent: Wednesday, September 06, 2006 2:46 PM
To: Vigersky, Robert A COL WRAMC-Wash DC
Cc: kkenney@usuhs.mil; ptackitt@usuhs.mil; Robert Goldstein; 'Mike Roy'
Subject: RE: SPACE FOR MY GENETICS STUDY

Dr. Vigersky,

I spoke to Patricia Tackitt and Kimbra Kenney within the Department of Neurology about the White Flint space. They seemed very optimistic regarding your requirements. Representatives from my division have seen this space and have been very impressed with the facility for outpatient research purposes. The total rent is approximately 4000/month and your contribution would be based on time and resources used, perhaps on the order of around 800/month, which is a very competitive rate in this area. They did request that you would use the space for a whole day/week as opposed to a half day. I've copied both on this email for further clarification of details.

Regarding the need for a USUHS Principal Investigator please let me know if this is still a requirement and I will address within my department.

Regards,

Kevin

Kevin Leary, MD
MAJ, MC

Appendix 4

-----Original Message-----
From: Richard Levine [mailto:rlevine@usuhs.mil]
Sent: Monday, August 14, 2006 3:35 PM
To: Stanila, Vlad V Dr WRAMC-Wash DC
Cc: Vigersky, Robert A COL WRAMC-Wash DC; Dana Thompson; John Baker; Laura Giberman; Margaret Pickerel; Micah Stretch; Steve Kaminsky; Trina Wadley [Contractor]
Subject: WRAMC Genetic Screening in Diabetes Protocol (WRAMC WU#06-13022)

Sir,

COL Vigersky's request to conduct the above WRAMC protocol on USUHS property was not approved by the University's School of Medicine administration.

As currently written, the protocol does not include a USUHS-billeted faculty member or permanent on-site researcher, a requirement for human subjects research conducted at the University.

Your protocol will be considered only after COL Vigersky has included a USUHS-billeted investigator who can serve as the responsible USUHS Principal Investigator and only after you have submitted an addendum to the WRAMC DCI for the addition of a USUHS collaborator and the DCI/HUC approval memo has been provided to the USUHS Office of Research. The local USUHS PI must fulfill the standard USUHS administrative requirements for a USUHS PI on a human subjects protocol, including the completion of USUHS Forms 3204 and 3208 as well as the required human

subjects protection training. You study team's training certifications should also be provided along with CVs for each WRAMC study team member and a copy of the WRAMC PI's medical license. All WRAMC team members should have completed their human subjects protections training within the past 12 months -- a requirement laid down the University's Human Subjects Protections Oversight Office at USD(P&R). Refresher training for study team members may also be required.

Please include a memo indicating the reasons why this study protocol cannot be wholly conducted at WRAMC and why you feel it needs to be conducted in USUHS space. We may need to provide your response to our Human Subjects Protections Oversight Office as well.

Please note that the protocol, if accepted by USUHS, will be reviewed by the USUHS IRB and no work may begin until the IRB has approved the study for conduct. Also note that a USUHS consent form will be required for subjects enrolled and tested at the University.

Rick Levine

Richard R. Levine, Ph.D.
Assistant Vice President
for Research
Uniformed Services University
4301 Jones Bridge Road
Bethesda, Maryland 20814-4799
Voice: (301) 295-3303
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rlevine@usuhs.mil

Appendix 5

>>> "Vigersky, Robert A COL WRAMC-Wash DC" <Robert.Vigersky@na.amedd.army.mil> 9/7/2006 9:34 AM >>> We will not be including any USUHS employees but it will be done in USUHS rental space. Dr. Kevin Leary has agreed to be the PI (pending his department's approval). The funds that we would use to sub-lease the space are also from Congressional funds, so I don't think that there should be any legal issues.

Robert A. Vigersky, M.D.,
COL MC
Director, Diabetes Institute
Walter Reed Health Care System

Genetic Screening in Diabetes: Candidate Gene Analysis for Diabetic Nephropathy
University of Hawaii
Lawrence Burgess, M.D. / Richard Arakaki, M.D. / Deborah Birkmire-Peters, PhD

INTRODUCTION:

The Genetic Screening in Diabetes: Candidate Gene Analysis for Diabetic Nephropathy project primarily launched a recruitment and data collection effort during the previous year. Having resolved issues arising from review of our protocol by the Institutional Review Boards of the three participating and sponsoring institutions, the Department of Defense, the University of Hawaii, and the St. Francis Medical Center (Dialysis and Transplant Centers), the first participant was seen in September of 2005. Additionally, other logistical considerations in conducting this study were implemented. Specifically, web-based subject registration, computerized questionnaire data collection, computerized blood processing and DNA extraction, and participant and primary care physician follow-up post screening were finalized. The effort at recruiting participants into this study, that is, subjects with physician-documented Diabetic Nephropathy (ESRD patients with dialysis or kidney transplant) with a first degree relative, have proved difficult and disappointing. Having had little success, we have embarked on strategies to enhance recruitment and increase enrollment. Our enrollment goal for this study still remains at a targeted number of 100 probands (individuals with Diabetic Nephropathy) and an average of 300 family members (parents and siblings) for this study. Without a large number of subjects, genetic and other characteristic information will not be meaningful.

BODY:

The initial recruitment effort that involved a direct approach to eligible patients in the Diabetes Clinic run by Dr. Arakaki yielded four families with one complete set of both parents and the patient with ESRD. The following describes the effort in review of the updated Diabetes Clinic database and recruitment effort:

- 50 patients with DM and ESRD were identified from the Diabetes Clinic
  - 10 were ineligible because of post-transplantation DM
  - 36 were eligible. Of these, 10 had had a transplant and 26 were on dialysis (2 PD).
- 40 eligible participants
  - 10 patients have not seen for the past 2 years. Letters of invitation to participate in the study have been sent to these patients and they will be called within a month.
- 30 participants were directly contacted
  - 9 patients were ineligible due to the absence of parents and siblings
  - 21 were eligible
    - 10 patients refused to participate (numerous reasons)
    - 7 patients are contemplating participation and have agreed to contact their parents and siblings
    - 4 participants were enrolled along with their family members

Of the three participants with DM and ESRD, two patients required hemodialysis and the other two patients were maintained on peritoneal dialysis. The first-degree relative of the four patients are listed below.

- Proband 1: Father (mother not scheduled, no siblings)
- Proband 2: Two sisters (parents deceased; 2 of 3 sisters, 1 not available)
- Proband 3: One brother (parents deceased; 1 of 2 siblings, 1 not available)
- Proband 4: Both father and mother evaluated

Additionally, other characteristics of interest are:
- Ethnicity: one Chinese, one Hawaiian, and two Japanese
- Proband ages ranged from 33 to 62 years
- All subjects had associated co-morbid condition of hypertension with DM

The recruitment effort at the dialysis and transplant centers of Saint Francis Medical Center (SFMC) had tremendous potential. The recent updated census of both centers revealed the following:

- 400 patients with hemodialysis
- 60 patients with peritoneal dialysis
- 200 patients with transplantation

An estimated 60% percent of these ESRD patients have diabetic nephropathy. Thus, the potential number of individuals eligible for screening is approximately 400 patients. However, based on our screening analysis (see above) from the Diabetes Clinic patient response, there will only be 50% eligible participants (no available parents or siblings) and of these eligible individuals another 50% interested. Thus, we are looking at recruiting from 125 individuals from the Dialysis and Transplant Centers. We will be able to reach our goal if everyone eligible participates. But again from our Diabetes Clinic analysis, we have found less enthusiasm to participate by the family members.

Our effort in working with the SFMC Centers has been quite disappointing. Because of the restriction imposed by the IRB due to HIPPA considerations, we are unable to directly contact potential individuals at the SFMC Centers. Thus, we have presented this study to the Director and the staff members of SFMC renal institute and asked for their assistance in recruitment. We initially made an oral presentation to the staff and have specifically asked for their help in passing out the brochure directly to patients, speaking to the patients on our behalf, and posting our flyers at multiple sites within the center. After a 3 month period post-presentation and seeking assistance from SFMC, there has been no contact from any patients from the Centers. We have gone back to discuss recruitment with the Director and the Medical Director of the Centers for their input and assistance, but still, no contact to date (as of August 2006).

In concert with the SFMC recruitment effort, we have embarked on a targeted informational campaign to patients with kidney disease. We set up an information booth during the National Kidney Foundation Fair in late May of 2006, and submitted an article for publication in the NKF Newsletter. Additionally, we also contacted Fresenius Medical Care, the other dialysis provider in the Islands to elicit their interest and assistance with recruitment. The primary Fresenius Dialysis Center in Honolulu accommodates approximately 300 patients. So far, their interest is high but formalizing the process to contact patients at their dialysis centers has been met with less enthusiasm. We are still working with the Director of Fresenius Medical Care to seek their assistance, which will require IRB approval.

Understanding the effectiveness of direct personal contact by physicians and staff members to recruit patients, new initiatives were spearheaded by Dr. Birkmire-Peters. She solicited the assistance of a leading nephrologist in the community. The nephrologist could easily recruit patients from her own practice which is nearly triple the number of patients with DM and ESRD than the Diabetes Clinic patient population. Additionally, a new staff member with primary responsibility for recruitment activities has been brought on board.

Lastly, we have discussed targeting patients with diabetic nephropathy but without ESRD, individuals who have chronic renal failure and impending dialysis. There are a fair number of these patients in the Diabetes Clinic as well as the nephrologists, and the etiological and genetic considerations are not any different than those who undergo dialysis. It may be easier to convince the subject and family members at this window in the progressive disease state to participate in this study. This consideration is included in the protocol and does not need additional IRB approval. However, specific characterization for identification and logistical processing will need to be considered.

KEY RESEARCH ACCOMPLISHMENTS:

- Institutional Review Board approvals obtained from: the University of Hawaii Committee on Human Studies, the Hawaii Pacific Health IRB (St. Francis Medical Center), and the Office of Regulatory Protections Human Subjects Research Review Board (USAMRMC).

- Web-based subject registration implemented
- Computerized questionnaire data collection programmed
- Computerized blood processing and DNA extraction implemented
- Participant and primary care physician follow-up post screening procedures implemented

REPORTABLE OUTCOMES:NONE

CONCLUSIONS:

This Genetic Screening in Diabetes: Candidate Gene Analysis for Diabetic Nephropathy project has encountered unforeseen difficulties in meeting the stated objectives. The previous hurdles of the prolonged IRB approval process have been overcome, but the restrictions on recruitment imposed by the Hawaii Pacific Health IRB (SFMC) has resulted in poor recruitment. The number of individuals screened and characterized is short of our goal and has delayed the timetable of the study. However, we have recently made number of overtures to overcome the recruitment difficulties, as well as, enacted new efforts to enhance recruitment of participants.

REFERENCES: NONE

APPENDICES: NONE